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A CYTOLOGICAL STUDY OF THE RESISTANCE OF APPLE VARIETIES TO GYMNOSPORANGIUM JUNIPERI-VIRGINIANAE [1]

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INTRODUCTION

A cytological study of the succession of pathologic events following the infection of susceptible and resistant host plants by the rust fungi affords a means of comparing the parasitic behavior of these organisms and contributing to the understanding of the mechanism of the resistance against their attack.

Since Biffen (*11*) [3] showed that the resistance of wheat to leaf rust, caused by *Puccinia triticina*, was a heritable character independent of the genetical factors responsible for anatomical features and Ward (*34, 35, 36*) stressed the concept of physiological resistance, many cytological studies have been made upon the rust diseases. The value of this avenue of approach is illustrated by the work upon the cereal rusts by Ward (*34*), Stakman (*33*), Allen (*1, 2, 3, 4, 5, 6*), Ruttle and Fraser (*32*), and others, who have presented a critical analysis of problems pertaining to the nature of rust resistance. The earlier work is reviewed by Allen (*1*), Rice (*31*), and Zimmermann (*39*) and need not be discussed here.

In accord with the behavior of rust fungi in general, the apple rust fungus, *Gymnosporangium juniperi-virginianae* Schw., shows a wide range in pathogenicity upon the foliage of the different varieties of its aecial host, the apple, *Pyrus malus* L. The degree of severity of the disease does not afford a rigid classification because many gradations exist between extreme susceptibility and apparent immunity. On susceptible varieties, the development of the fungus culminates in the production of the aecial stage. On the other hand, there are varieties upon which no macroscopic signs of infection appear. Between these two extremes lie those resistant varieties upon which the fungus becomes established but does not develop very far beyond the incipient stages. In come cases, various types of flecking are produced, while in others the lesions may be well defined, and minute or aberrant pycnia may appear.

Previous studies of the nature of rust resistance in apple varieties have been confined to secondary considerations supplementary to the broader epidemiological and pathological aspects of the disease. From field observations of the occurrence and severity of infection upon different varieties, Reed and Crabill (*30*) believed resistance to

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[3] Reference is made by number (italic) to Literature Cited, p. 594.

be of two types: (1) The prevention of infection, due to the mechanical, physical, or chemical nature of the epidermis, and (2) the incompatibility of the host and parasite after infection had occurred, resulting in the production of an aberrant lesion. They described briefly the pathological histology of apple rust infections in a susceptible host and made microscopic examinations of infections in the leaves of Arkansas, a resistant variety upon which abnormal lesions occurred. However, the sequence of events in the development of these infections and the cytological details involved were not reported.

Leaves of susceptible varieties become resistant with age. Giddings and Berg (*20*) and Reed and Crabill (*30*) attached much significance to this phenomenon as a factor in the regulation of the severity of the disease. They discussed the nature of resistance in mature leaves but did not give factual evidence to explain it. Miller (*27*) observed the occurrence of normal infections upon mature leaves of susceptible varieties that had been wounded mechanically prior to inoculation. This led to the interpretation that the resistance shown by mature leaves had a morphological basis.

The sporidial germ tubes of *G. juniperi-virginianae* commonly infect apple leaves through the ventral epidermis. Reports of dorsal-surface infections are at variance. Coons (*14*) implied that infection of the lower epidermis occurred but stated, however, that upper-surface inoculations were more successful. The experiments of Giddings and Berg (*20*) showed that no infection followed inoculation of the lower epidermis. On the other hand, Weimer (*38*) reported that infection could take place upon either surface. Dorsal surface infection, however, had no effect upon the normal orientation of the pycnia and aecia. In the literature dealing with apple rust, further information with a bearing upon the nature of resistance has not been found.

From the above accounts, it is apparent that little is known concerning the parasitic behavior of the apple rust fungus or the nature of apple rust resistance. In view of these problems, a detailed cytological study of rust infections upon four apple varieties, each differing in its reaction to the fungus, was undertaken. The results of these studies are presented in this paper.

MATERIALS AND METHODS

Four varieties of apples were used in these studies as follows: Wealthy, susceptible; Yellow Transparent, moderately resistant; Fameuse, resistant; and Baldwin, very resistant. Two-year-old nursery trees were placed in galvanized-iron containers in about 10 kg of soil adjusted and held at 70 percent of the maximum water-holding capacity. After being rooted in a cool basement, they were placed on the greenhouse bench. One shoot was allowed to develop on each tree. When the shoots were about 18 inches long and showed 14 to 20 leaves, they were inoculated.

Rust material for inoculation was collected near Madison, Wis. Branches of red cedar (*Juniperus virginiana* L.) bearing abundant rust galls with extended telial sori were brought into the laboratory and the cut ends of these twigs were kept in water until the material was used.

Several methods of inoculation were tried with varying results. The following method proved most useful and was employed through-

out: The rust galls were moistened in a spray of water for 4 hours, after which the telial sori were well gelatinized. A few hours later, when sporidial discharge was taking place rapidly, the galls were suspended in a glass lamp chimney which was held above the leaves to be inoculated in such a way that the sporidia would fall upon the upper surfaces of the leaves. One lot of leaves of the Wealthy variety was inoculated on the lower surface. Each shoot was exposed for a 15-minute period. The inoculated trees then were placed in a moist chamber, described by Keitt and Jones (*21*), at 14° C. for 36 hours, after which they were returned to the greenhouse bench.

Question may arise regarding the purity of the rust culture used. Recently, Bliss (*12*) reported the existence of physiologic forms in *G. juniperi-virginianae* collected from widely separated localities. The writer believes, however, that only one physiologic form was used in these studies because the material was collected from cedar trees in an isolated group and inoculation experiments upon many apple varieties with similar cultures during the past 3 years have shown uniform pathogenicity.

After inoculation, collections were made at daily intervals for the first week and at longer intervals thereafter for the next 2 weeks. One earlier collection was made after a 14-hour period. At the time of inoculation, a bit of string was tied about the petiole of the youngest leaf in order to facilitate identification of the inoculated leaves after further growth had occurred. Material always was taken from the fourth or fifth leaf below the "stringed" leaf to insure uniformity. One lot of Wealthy leaves, which had attained approximately full size, and another group about 3 weeks older were collected in an attempt to gain evidence pertaining to the relationship of maturity to resistance. Fixations were made in formal-chrom-acetic mixture, in Flemming's medium and weak mixtures, and in the formal-acetic-alcohol fixative. Flemming's weak fluid was the most useful for young material, whereas the formal-chrom-acetic mixture was best for the later stages. The butyl alcohol method of Zirkle (*40*) was used for dehydration and embedding in paraffin. Sections were cut 5μ and 6μ thick. A modification of Flemming's triple stain, consisting of safranin, gentian-violet, and fast green, gave satisfactory results.

A modification of the cleared-leaf method of Peace (*29*) was well adapted to a superficial study of sporidial germination and the early stages of penetration because it afforded a means of observing the germinating sporidia in toto upon relatively large areas of leaf surface. Bits of leaf were collected at 7, 14, and 24 hours after inoculation, fixed in acetic-alcohol mixture, cleared in chloral hydrate, and stained with acid fuchsin in lactophenol.

MACROSCOPIC OBSERVATIONS ON LIVING LEAVES

On the immature leaves of Wealthy, inoculated upon the ventral surface, pale yellow spots 1 mm in diameter appeared on the seventh day. The lesions increased in size to about 2 mm during the next 2 days and became orange in color. Pycnia appeared on the thirteenth day and pycnial exudation occurred on the fifteenth day. The heaviest infection appeared on the fourth, fifth, and sixth leaves below the stringed leaf. At the time of inoculation, these leaves were rapidly expanding and had not reached full size. No infection was

found below the tenth leaf, indicating that the older, matured leaves had become resistant. Wealthy leaves, inoculated upon the dorsal surface only, showed an occasional spot appearing on the twelfth to the fourteenth day. These lesions developed in the usual manner. The prolonged incubation period suggests that the parasite may have had some difficulty in becoming established.

On the leaves of Yellow Transparent infection appeared on the ninth day as indistinct pale yellow spots about one-quarter millimeter in diameter. A few days later the infected leaves showed a pale yellow mottling with few well-defined lesions. On the eighteenth day an occasional pycnium could be seen in the regions where the lesions were more clearly outlined. These pycnia were minute and failed to rupture the epidermis or exude.

On the leaves of Fameuse, the first signs of infection appeared on the twelfth day as tiny flecks which were barely visible. No further development was evident.

On the leaves of Baldwin, no macroscopic signs of infection showed throughout the 3-week period the trees were under observation. These external appearances indicated complete resistance.

EARLY STAGES OF INFECTION

CLEARED-LEAF STUDY

The phenomena of sporidial germination and of the early stages of penetration, as observed on cleared leaves, appeared to be similar on all varieties. Counts made on germination of sporidia, formation of appressoria and the appearance of infection hyphae showed insignificant differences (taole 1).

It is not probable that these data are vitiated to any appreciable extent by the removal of some of the sporidia by the reagents used in clearing and staining the leaves. Previous experience of the writer indicates that, when the sporidia become lodged upon the leaf surface, they cling tenaciously.

After the 7-hour period, nearly all of the sporidia had germinated. The germ tubes in no case exceeded in length the diameter of the sporidium. There was no indication that the tips of the germ tubes were attached to the leaf surface.

TABLE 1.—*Data on germination of 100 sporidia, formation of appressoria, and development of infection hyphae on four apple varieties*

Variety	Age of leaf	Surface inoculated	Percentage development after stated periods								
			7 hours			14 hours			24 hours		
			Germinated	Appressoria	Infection hyphae	Germinated	Appressoria	Infection hyphae	Germinated	Appressoria	Infection hyphae
Wealthy	Young	Ventral	91	0	0	98	89	8	97	97	97
	do	Dorsal	98	0	0	95	91	21	97	97	97
	Old	Ventral	88	0	0	99	83	22	96	96	96
	do	Dorsal	92	0	0	96	89	10	100	100	100
Yellow transparent.	Young	Ventral	94	0	0	95	84	11	99	99	99
Fameuse	do	do	95	0	0	100	92	15	97	97	97
Baldwin	do	do	93	0	0	95	86	5	96	96	96

After the 14-hour period, the process was distinctly further advanced. Most of the germ tubes had formed appressoria, the term "appressorium" being used in a broad sense throughout to describe the swelling of the tip of the sporidial germ tube adherent to the leaf surface. The germ tubes were seldom longer than the diameter of the sporidium and, in many cases, they were so short that the appressoria appeared to be attached directly to the sporidia. The appressoria were not well defined. They appeared only as swellings of the germ-tube tips. A few of these appressoria showed a minute porelike

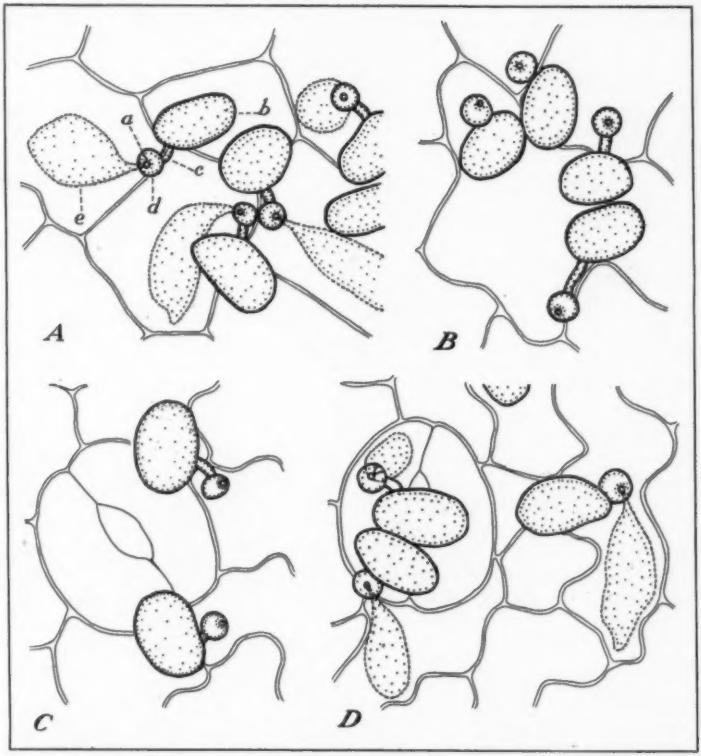


FIGURE 1.—Camera-lucida sketches of sporidial germination and host penetration from cleared leaves examined in toto: *A*, ventral surface of young Wealthy leaf, pore of infection hypha (*a*), sporidium (*b*), germ tube (*c*), appressorium (*d*), primary hypha in epidermal cell (*e*); *B*, ventral surface of old Wealthy leaf; *C*, dorsal surface of old Wealthy leaf; *D*, dorsal surface of young Wealthy leaf. Approximately × 775.

structure, about 1μ to 2μ in diameter, in the wall in contact with the leaf. This phenomenon suggested the development of an infection hypha. There was, however, no apparent change of the underlying host tissues to indicate that the fungus had entered.

After the 24-hour period, all of the germ tubes were firmly attached to the leaf surface and characteristic pores were clearly visible in all of the appressoria. In some cases the appearance of a large primary

hypha in the underlying epidermal cell indicated that penetration had occurred, but this manifestation was too indistinct to be relied

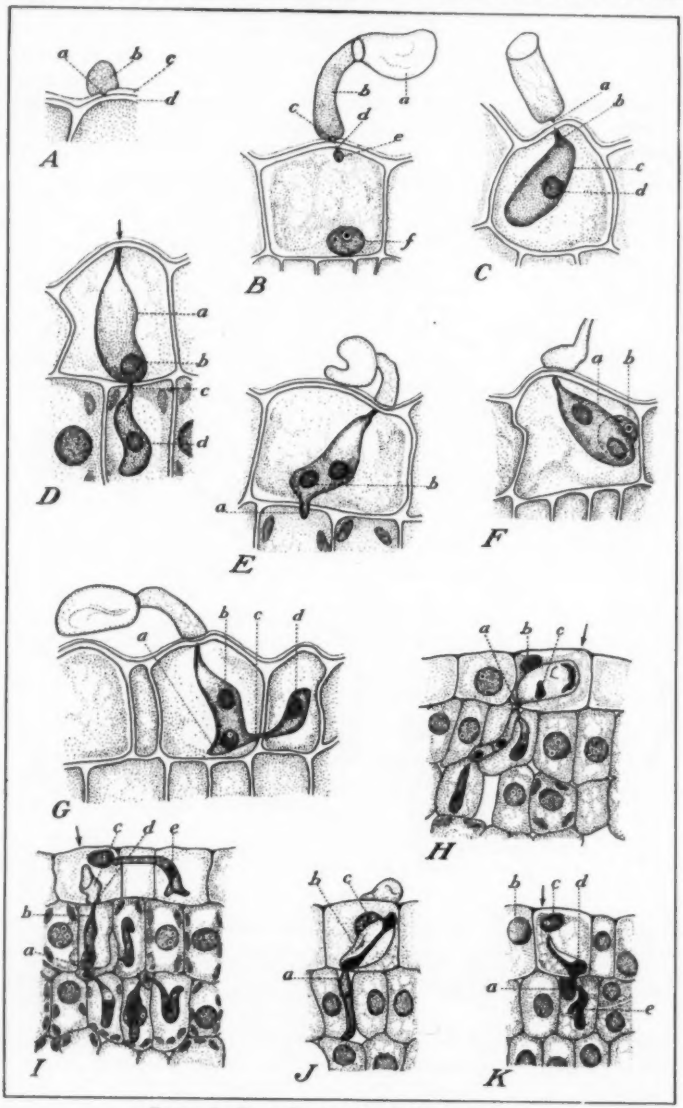


FIGURE 2.—For explanatory legend see opposite page.

upon as an absolute criterion. The appearance of the 24-hour stage of development upon both upper and lower surfaces of young and old Wealthy leaves is shown in figure 1.

This study was not carried further because a technic was not found which would satisfactorily stain the fungus within the leaf.

PHENOMENON OF PENETRATION

The fungus entered the immature leaves of all four varieties and in all cases the process was essentially the same. The germ tubes emerged from the sides of the sporidia and curved obtusely, sometimes almost at right angles, down to the leaf surface, where the germ-tube tips swelled slightly into small appressoria which were firmly attached to the cuticle (fig. 2, *E*, *G*). Waterhouse (*37*), working with *Puccinia graminis* on the barberry, reported that germ tubes were not always formed. In case they were not, a short beaklike infection hypha was put out from one end of the sporidium itself and dented the cuticle. Allen (*7*), also working with *P. graminis*, observed that the sporidia directly produced a beak which pierced the cuticle and outer epidermal wall. After 14 hours, the lower wall of the appressorium of the apple rust fungus showed a short protrusion partly penetrating the cuticle (fig. 2, *A*). At that time, the sporidium was nearly evacuated and the germ tube was filled with dense protoplasm. Nuclei could not be seen.

The earliest stage of penetration is shown in a 24-hour infection in figure 2, *B*. The minute pore is shown at *c* and from it the infection hypha (*d*) can be followed part way through the upper wall of the epidermal cell to the young primary hypha (*e*) in the cavity of the cell. The host nucleus remains lying in the bottom of the cell at *f*. In no case could the infection hypha be traced all the way through the cuticle and the cell wall, there being a short space about half-way through the barrier, where it could not be seen. This suggests that it was ultramicroscopic or was not sufficiently stained in that portion to be visible. The facts that there was no apparent alteration of the cuticle or epidermal cell wall at the point of entry and that the cuticle was often indented indicate mechanical pressure as the principal factor involved in penetration. This is in agreement with Waterhouse (*37*). Weimer (*38*) described and figured one case where a sporidial germ tube of *G. juniperi-virginianae* penetrated

EXPLANATORY LEGEND FOR FIGURE 2

A.—Early stage of penetration on Wealthy, 14 hours after inoculation. The beak (*a*) of the appressorium (*b*) is indenting the cuticle (*c*) but has not penetrated the epidermal cell wall (*d*). × 1,070.

B.—Penetration on Wealthy, 24 hours after inoculation. Empty sporidum (*a*), germ tube (*b*), penetration pore (*c*), infection hypha (*d*), young primary hypha (*e*), and nucleus of epidermal cell (*f*). × 1,070.

C.—Early stage of infection on Wealthy, 24 hours after inoculation. The primary hypha (*c*) contains one nucleus (*d*) and remains attached to the infection hypha (*a*) by a slender neck (*b*). × 1,070.

D.—One-day-old infection on Wealthy. Arrow indicates point of entry. Primary hypha (*a*) is slightly evacuated and contains one nucleus (*b*); secondary branch has penetrated palisade cell at *c* and contains one nucleus (*d*). × 1,070.

E.—One-day-old infection on Wealthy. Young secondary branch (*a*) has penetrated palisade cell and one of the nuclei (*b*) is ready to pass into it. × 1,070.

F.—One-day-old infection on Wealthy. The primary hypha (*a*) is binucleate; epidermal cell nucleus in attendance at *b*. × 1,070.

G.—One-day-old infection on Wealthy. The primary hypha (*b*) sent a branch (*d*) into an adjoining epidermal cell at *c*; another branch appears as a beak (*a*) and a nucleus is ready to pass into it. × 1,070.

H.—Two-day-old infection on Yellow Transparent. An arrow indicates the point of penetration. The nucleus (*c*) of the primary hypha is dead; a secondary branch entered the palisade intercellularly at *a*; the epidermal cell nucleus (*b*) is degenerating. × 700.

I.—Two-day-old infection on Wealthy. An arrow indicates the point of penetration. The primary hypha (*c*) sent a branch (*e*) into an adjoining epidermal cell and another branch (*b*) into a palisade cell at *d*; mycelium becoming intercellular at *a*. × 700.

J.—Two-day-old infection on Fameuse. Primary hypha (*b*) has collapsed; host cell nucleus (*c*) is degenerating; intercellular branch (*a*) is developing feebly. × 700.

K.—Two-day-old infection on Baldwin. An arrow indicates the point of penetration. Primary hypha (*d*) and intracellular secondary branch (*e*) are dead; host nuclei (*a* and *c*) are degenerating; host nucleus (*b*) is slightly enlarged. × 700.

the epidermis of a Wealthy apple leaf and passed about two-thirds of the distance through the epidermal cell 7 hours after inoculation. This must have been an unusual case. Examination of several hundred germinating sporidia by cleared leaf and cytological methods failed to show that any had penetrated the epidermis completely after 14 hours.

Other 24-hour infections showed stages considerably advanced over the one described above. The instance in figure 2, *C*, shows that the primary hypha (*c*) has enlarged into a turgid, saccate body which remained joined to the infection hypha (*a*) by a slender neck (*b*). The hypha contains one nucleus (*d*) and is filled with dense cytoplasm. The nucleus is probably the one originally contained in the sporidium and has not yet divided. Allen (*9*) reported that in *Puccinia coronata* the sporidial nucleus divided before penetration and that the primary hypha, therefore, contained two nuclei. Two nuclei were also found in the primary hypha of *P. graminis* (*7*) but whether or not division took place before entry was not investigated.

In these preparations of the younger stages of infection and of the older ones as well, the fixations obtained did not afford a clear interpretation of the relationship between the membranes of the host cell and the fungus. It was impossible to determine whether the host-cell cytoplasm was invaginated by the primary hypha or haustoria or actually penetrated by them. The views of other workers on this point are reviewed by Rice (*31*).

During the process of penetration and the expansion of the primary hypha of the apple rust fungus, there was no apparent change in the staining reaction of the parasitized epidermal cell. Further development, however, depended upon the degree of resistance of the host. Consequently, the reactions in each variety are reported separately.

DEVELOPMENT OF INFECTION ON THE FOUR VARIETIES

WEALTHY

The establishment of the fungus in the leaf tissues of Wealthy presented a picture of almost complete compatibility between the two organisms. This condition existed until pycnial formation when distinct changes accompanied the fruiting activities of the parasite.

The behavior of the young primary hyphae in the epidermal cells was simple and afforded trustworthy interpretation. Soon after the primary hypha attained full size, the nucleus divided (fig. 2, *F*). These nuclei were similar in appearance to the parent nucleus. No septa appeared in the primary hypha and the structure remained simple throughout its existence. In *Pucinnia graminis* (*7*), *P. coronata* (*9*), and *P. triticina* (*8*) a more complicated process, similar in all cases, was reported by Allen. Nuclear and cell division occurred until from 3 to 6 cells were produced. The primary hypha increased in length and branches of secondary hyphae arose from each cell. In *Melampsora lini* (*10*), the primary hypha consisted of several uninucleate cells, irregularly branched. Palisade penetration occurred at many points.

The number of secondary hyphal branches of the apple rust fungus seemed to be dependent upon the behavior of the first one formed. In figure 2, *G*, a branch (*d*) has penetrated into an adjoining epidermal

cell at *c*, and contains one of the daughter nuclei of the first division. Another nuclear division has occurred in the primary hypha and one of the nuclei is ready to pass into a new branch which appears as a beak at *a*.

The formation of only one secondary branch, penetrating the palisade tissues directly, was the most common occurrence. In figure 2, *D*, a branch (*d*) has entered the palisade cell beneath and a nucleus has entered, leaving one (*b*) in the primary hypha. The primary hypha has the appearance of being evacuated and the branch is filled with dense protoplasm. In *E*, the branch appears as a peg-like outgrowth (*a*) which is penetrating into the underlying palisade cell and a nucleus (*b*) is ready to pass into it.

Palisade penetration occurred without any apparent retardation of apical growth. There was no constriction of the hypha at the point of cell-wall penetration and no visible alteration of either the cellulose or middle lamellar portions of the walls. Leach (*23*) working with *Colletotrichum lindemuthianum*, observed that the primary hypha bent, and that the advancing tip swelled, due to the retardation of apical growth during cell-wall penetration. He also stated that the hole made through the cell wall was very small and that the mycelium was constricted at that point. Allen reported that the opening in a cell wall through which a branch of the secondary hypha of *Puccinia graminis* (*7*) passed was too small to be seen, but that in *P. triticina* (*8*) it was large.

Similar to *Puccinia graminis* (*7*), the early development of the secondary hyphae of *Gymnosporangium juniperi-virginianae* depended upon whether or not they entered palisade cells or intercellular spaces. If they were intercellular, the hyphae immediately expanded into characteristic strands of mycelium. If they were intracellular, they assumed the appearance of large haustoria, each connected to the point of entry by a slender neck.

A 2-day-old infection is shown in figure 2, *I*. An arrow points to the place of entry. The primary hypha (*c*), which was partly lost in sectioning, is slightly evacuated. One branch (*e*) (the connection was lost in sectioning) has penetrated the adjoining epidermal cell. This secondary hypha contains one large nucleus and is forming two new branches at the distal end. Palisade penetration occurred intracellularly at *d*. The penetrating hypha (*b*), is somewhat swollen, is unbranched, and consists of only one cell. It has passed entirely through the cell and emerged into an intercellular space at *a*. From here on, the mycelium is regularly intercellular with the formation of characteristic uninucleate haustoria.

While still in the palisade region, the hyphae exhibited difficulty in passing through the compact tissues. The hyphal tips, viewed longitudinally, appeared as wedges prying the cell walls apart. The fact that there was no splitting apart of the walls in advance of the hyphal tips precluded the view that the fungus might have some solvent action upon the middle lamella. It seemed that considerable pressure would have to be exerted to force a passage mechanically, but that is probably what actually occurred. Allen (*9*) made similar observations upon *Puccinia coronata* where the early development of the fungus regularly takes place in the compact subepidermal region.

The haustoria entered the cells from the intercellular hyphae without the formation of attachment organs. The only stimulus neces-

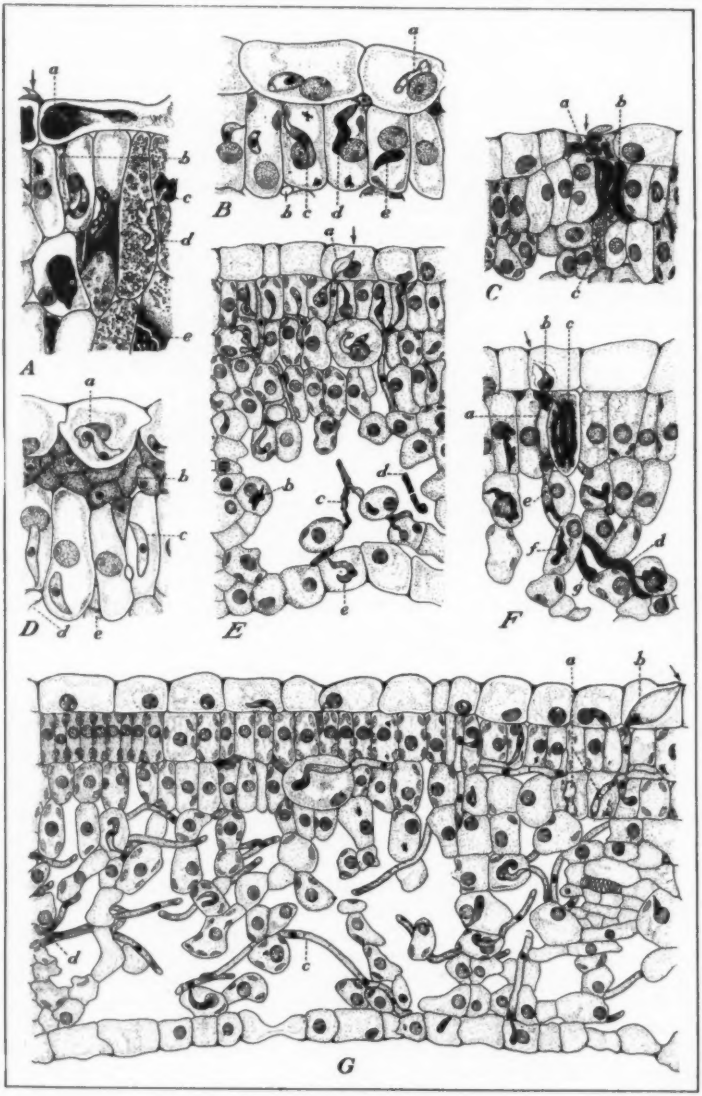


FIGURE 3.—For explanatory legend see opposite page.

sary for haustorium formation seemed to be close contact of the tip or side wall of a hypha with the host-cell wall. A nucleus was usually found in the hypha near the point of haustorium formation and the

haustoria were regularly uninucleate. It seems reasonable to assume, therefore, that a nuclear division took place in the hypha and that one of the daughter nuclei passed into the haustorium, although the process was not observed. The pore of entry was about 2μ to 3μ in diameter and could be seen clearly. There was no apparent change in the cell wall at that point. The main body of the haustorium was connected to the intercellular hypha by a slender neck. In all of the younger infections the haustoria were one-celled and uninucleate, without any branching. The haustoria generally were filled more densely with protoplasm than the intercellular hyphae and took up the violet of the triple stain heavily.

Growth of the mycelium was accelerated greatly after the second day. A median section of a 4-day-old lesion is shown in figure 3, *G*. An arrow indicates the point of entry, and the primary hypha, turgid in appearance and containing one nucleus, is shown at *b*. Intercellular penetration of the palisade occurred and the secondary hypha immediately branched and ramified the local palisade region to a limited extent. Simple, uninucleate haustoria occupy most of the palisade cells in the region of penetration and slight depletion is evident in the reduction in the number of plastids. Two cells at *a* show disorganization of their protoplasts. The host nuclei in the parasitized cells were usually in contact with the haustoria but only because of the vigorous expansion of the haustoria. There was no apparent movement of the nuclei in response to the presence of the fungus. Haustoria were not seen which encircled the nucleus or changed its shape due to contact or the application of pressure against it. In fact, all of the host nuclei retained their normal structure and staining reaction.

After gaining access to the large intercellular spaces of the spongy parenchyma region, the fungus spread rapidly. Long hyphae appear as runners (fig. 3, *G*, *c*, *d*) which have already spread a distance of about 200μ from the point of entry. These runners are sparsely branched, contain dense protoplasms, and have widely separated septa. The cells are uninucleate. Occasional parenchyma and lower epidermal cells along their path contain characteristic haustoria which, no doubt, help to supply food for this rapid growth.

Spread of the mycelium was much less rapid in the palisade than in the spongy parenchyma. Haustoria, however, were more numerous

EXPLANATORY LEGEND FOR FIGURE 3

A.—Eight-day-old infection on Fameuse. An arrow points to the remains of the appressorium. Epidermal cell (*a*) contains disintegrated primary hypha and collapsed host-cell contents; intercellular hypha (*b*) is vacuolate; degenerating host-cell protoplast invaginated by dead haustorium (*e*); haustorium at *d* empty and one at *c* killed back from tip. × 650.

B.—Seven-day-old infection on Yellow Transparent. Dead haustoria (*d* and *e*) occupying living cells, normal haustorium (*c*), empty haustorium (*a*), evacuated hypha (*b*). × 650.

C.—Four-day-old infection on Baldwin. An arrow indicates the point of penetration. The epidermal cell of entry has collapsed and contains the remains of the dead primary hypha. Haustorium (*a*) in a living cell died before it fully expanded; palisade cells (*b*) are dead; host cell (*c*) disintegrating. × 440.

D.—Pycnial initiation on Wealthy, 9 days after inoculation. Mass of hyphae (*b*) in subepidermal region; haustoria (*a* and *c*) and intercellular hyphae (*d* and *e*) becoming evacuated; host cells impoverished. ×650.

E.—Four-day-old infection on Yellow Transparent. An arrow indicates the point of penetration. Primary hypha (*a*) partly collapsed; haustorium (*b*) and hypha (*d*) are dead; hypha at *c* degenerating; normal haustorium in lower epidermal cell at *e*. × 440.

F.—Four-day-old infection on Fameuse. An arrow indicates the point of penetration. Primary hypha (*b*) dead; intercellular branch (*a* and *e*) degenerating; host cell (*c*) contains the remains of a dead haustorium and of its own collapsed protoplast; dead haustorium (*f*) in a living cell; advancing hyphae (*d* and *g*) densely filled with protoplasm. × 440.

G.—Four-day-old infection on Wealthy. An arrow indicates the point of penetration. Primary hypha (*b*) uninucleate and turgid; vacuolate haustoria (*a*) occupy cells with broken down protoplasts; stolonlike branches in spongy parenchyma region at *c* and *d*. × 440.

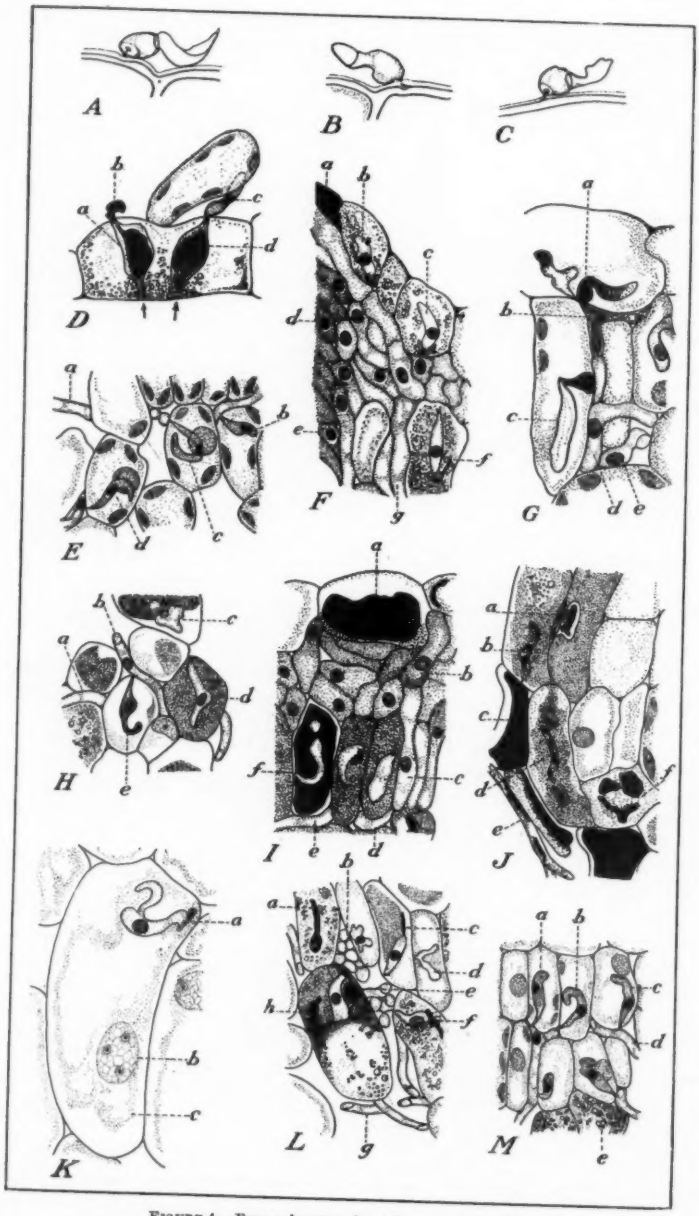


FIGURE 4.—For explanatory legend see opposite page.

and in a few cases appeared in the upper epidermal cells. This is not in agreement with Reed and Crabill (*30*) who reported that they did not find haustoria in the epidermal cells of apple rust infections.

From the fourth to the tenth day, there was very little change in host-parasite relations. The lesions continued to spread in all directions, especially in the spongy parenchyma region. In the older parts of a 9-day-old infection (pl. 1, *A*) nearly every host cell contained an haustorium. Depletion of the cytoplasm of these cells was still slight, although many of the plastids had disappeared. The margin of the lesion appeared similar to the margin of the 4-day-old lesion. Near the center of the infection (fig. 3, *D*), hyphae had begun to accumulate in the subepidermal region preparatory to pycnial formation. These hyphae were filled with dense cytoplasm (*b*) whereas the haustoria (*a* and *c*) and other vegetative hyphae (*d* and *e*) appeared to be nearly empty.

After the pycnia had ruptured the epidermis, the activity of the host and of the parasite showed decidedly different trends. A photomicrograph of a 15-day-old infection (pl. 1, *C*) shows the general character and location of the reactions.

In the spongy parenchyma region below the pycnia, hypertrophy of the cells caused the obliteration of the large intercellular spaces and the collapse of a large portion of the lower epidermis. A representative cell from this region is shown in figure 4, *K*. It is approximately four times normal size and contains a uninucleate, branched haustorium at *a*, which is almost empty. The host nucleus (*b*) is approximately twice the normal size and is stained very faintly, giving the impression that the reticulum was loosening. The lumen of the cell otherwise appears empty except for a thin strand of cytoplasm (*c*). The intercellular hyphae appeared to be empty except for their nuclei.

EXPLANATORY LEGEND FOR FIGURE 4

A.—Penetration failure upon old Wealthy leaf, 3 days after inoculation. Cuticle indented and appressorium partly raised caused by the pressure of the infection hypha which has failed to penetrate. × 850.

B.—Penetration failure upon old Wealthy leaf, 3 days after inoculation. Infection hypha has penetrated the cuticle but failed to puncture the epidermal cell wall. × 850.

C.—Penetration failure upon old Wealthy leaf, 3 days after inoculation. Infection hypha penetrated the cuticle and flattened out against the epidermal cell wall; the appressorium is slightly raised. × 850.

D.—Dorsal surface of young Wealthy leaf, 5 days after inoculation. Arrows indicate the point of penetration in two separate infections. Primary hyphae (*a* and *d*) are dead and their secondary branches (*b* and *c*), after having emerged into large intercellular spaces, have also succumbed. × 850.

E.—Tissues from the marginal region of a 15-day-old infection on Wealthy. Normal haustoria (*b*, *c*, and *d*) occupying living cells; vacuolate hypha (*a*). × 700.

F.—Margin of pycnium in a 15-day-old infection on Wealthy. Dead epidermal cell (*a*); evacuated haustoria (*b* and *c*) occupying broken-down epidermal cells; bases of pycnosporophores (*d* and *e*) densely filled with protoplasm; intercellular hyphae (*g*) nearly empty; haustorium with dead tip (*f*) lying in a degenerated host-cell protoplast. × 700.

G.—Portion of an 18-day-old lesion in a Wealthy leaf of mature age at the time of inoculation. Haustoria (*a* and *c*) partly empty and dead; hyphae (*d* and *e*) vacuolate but still alive; hypha (*b*) degenerating. × 1,070.

H.—Tissue from the center of a 15-day-old infection on Yellow Transparent. Vacuolate hypha (*a*), normal hypha and haustorium (*b* and *c*), empty haustorium (*c*), injured haustorium occupying degenerating host cell protoplast at *d*. × 700.

I.—Pycnial initiation in 15-day-old infection on Yellow Transparent. Collapsed epidermal cell (*a*), empty haustorium in dead host cell (*f*), vacuolate hyphae (*c*, *d*, and *e*), pycnial initials (*b*). × 700.

J.—Margin of 15-day-old infection on Fameuse. Dead haustoria occupying disintegrated host cells (*a*, *b*, *d*, and *f*), with invagination of cytoplasm at *a*; dead hypha (*e*); collapsed host cell (*c*). × 700.

K.—Portion of spongy parenchyma tissue beneath a pycnium from a 15-day-old infection on Wealthy. A uninucleate haustorium (*a*), thin film of host-cell cytoplasm (*c*), hypertrophied host nucleus (*b*). × 700.

L.—A portion of palisade tissue beneath a pycnium in a 15-day-old infection on Wealthy. Dead haustorium (*a*), haustoria (*c*, *f*, and *h*) showing partial collapse at the tips, host nucleus (*e*) disintegrating, intercellular hyphae (*b* and *g*) evacuated. × 700.

M.—Tissue from marginal zone of a 15-day-old infection on Yellow Transparent. Vacuolate hypha (*d*), normal haustoria (*a*, *b*, and *c*) occupying living cells, degenerating host cell (*e*). × 700.

The palisade cells in the vicinity of the pycnia were crushed and their protoplasts were being broken down. A portion of palisade tissue beneath a mature pycnium is shown in figure 4, *L*. The host cells are slightly hypertrophied and are disorganized. Their contents appear mostly as aggregates of small globules which were not positive to any of the stains used. This indicates coagulation of the protoplasm. A host nucleus in the process of disintegration is shown at *e*. Haustoria are also in progressive stages of collapse. The one at *a* is dead. Those at *c*, *f*, and *h* have been killed back from the tip and their basal portions, aside from the presence of large nuclei, are empty. The intercellular spaces are nearly filled with empty hyphae (*b*).

The upper epidermal cells also show degeneration due to the now aggressive attack of the fungus. In figure 4, *F*, a group of these cells bordering a pycnium show prominent haustoria at *b* and *c* partly surrounded by disintegrated host-cell protoplast. The only epidermal cells to collapse completely, however, are those near the ostioles of the pycnia (*a*). The pycnosporophores (*d* and *e*) are densely filled with protoplasm and the bordering hyphae are nearly empty.

In the outer zone of infection, congeniality between the host and parasite continued to exist although marginal extension of the mycelium had practically ceased. The runners, typical of younger infections, were absent. A group of cells near the margin of a 15-day-old lesion are shown in figure 4, *E*. Haustoria at *b*, *c*, and *d* are uninucleate and contain dense cytoplasm. A vacuolate, intercellular hypha is shown at *a*. The host cells are little affected. Distinct plastids still remain.

YELLOW TRANSPARENT

Yellow Transparent was intermediate in its reaction to rust infection. The fungus became established and was able to maintain congenial relations with the host long enough to continue its gradual spread. Finally, development ceased altogether and the whole lesion collapsed before pycnia matured.

A 2-day-old infection is shown in figure 2, *H*. An arrow points to the place of entry. The primary hypha is swollen and contains one nucleus (*c*) which is dead, and a mass of dead cytoplasm at the proximal end. Otherwise, it is almost empty and apparently has ceased activity. The host nucleus at *b* is degenerating. It seems probable that the primary hypha succumbed first owing to its inability to endure the unfavorable medium surrounding it, and that the partial collapse of the host nucleus is a result of the diffusion of deleterious or toxic materials from the dead fungous protoplasm. Intercellular penetration of the palisade occurred at *a* and the secondary hypha has sent out two haustoria, which have fully expanded.

A 4-day-old infection (fig. 3, *E*) shows decided advancement. An arrow indicates the point of entry. The primary hypha (*a*) has partly collapsed and is empty. Palisade penetration occurred and the mycelium ramified the local palisade region sending typically normal haustoria into many of the cells. The relation here is congenial with very little depletion of the host cells. The mycelium has developed feebly in the spongy parenchyma. The long runners, common in infections in Wealthy leaves, are absent and the whole lesion is not more than 100μ in diameter. A dead hypha is shown at *d* and one

in a partial state of collapse at *c*. An haustorium (*b*) is dead but its host cell shows no deleterious effects. Contact has been made with the lower epidermis at *e*. None of the host cells showed any degeneration or drastic reaction to infection, even though some of the hyphae were dead and collapsed.

Marginal spread of the fungus continued slowly until about the ninth day, when slight hypertrophy and degeneration of the host cells in the center of the lesion began. Rapid break-down of nearly all of the parasitized host-cell protoplasts followed. Only a narrow zone at the margin of the lesion showed a compatible relationship between the parasite and the host. A photomicrograph of a 15-day-old infection (pl. 1, *B*) shows that nearly all of the host-cell protoplasts in the upper-epidermis, lower-epidermis, and mesophyll tissues are degenerating or have already disintegrated.

The collapse of the host cells mentioned above followed closely upon the gradually increasing injury to the fungus. A section of a 7-day-old infection (fig. 3, *B*) shows that the palisade cells near the center of the lesion are still apparently unharmed except for a depletion of cytoplasmic substances and plastids. The host-cell nuclei still retain their normal structure and staining reaction. On the other hand, haustoria at *d* and *e* are dead. Their contents are coarsely granular and heavily stained with safranin. The haustorium at *c* has retained its normal healthy appearance.

The details of the disorganization which followed are represented in three illustrations taken from different regions of 15-day-old infections. The palisade and upper epidermal tissues in the central portion appear in figure 4, *I*. Large, densely filled hyphae (*b*) are accumulating in the subepidermal region preparatory to pycnial formation. The intercellular hyphae at *c*, *d*, and *e* are becoming evacuated, as well as the haustoria occupying collapsed palisade cells. None of the host cells is now alive and it is of special interest to note that the hyphae lying adjacent to them appear to be unharmed. A group of cells from the spongy parenchyma region (fig. 4, *H*) show widespread disintegration of host cells. Haustoria (*c* and *d*) are nearly empty and are embedded in the broken-down host-cell protoplasts. The hypha at *b* and the haustorium at *e*, however, still retain a healthy appearance. Tissue from the marginal zone is shown in figure 4, *M*. The haustoria (*a*, *b*, and *c*) are fully expanded and the invaded cells appear to be only slightly affected. The adjoining cell (*e*), however, shows hypertrophy and degeneration.

FAMEUSE

The leaves of Fameuse were resistant to rust infection. The lesions were usually confined to small regions in the upper epidermis, palisade, and spongy parenchyma tissues. Very seldom did the hyphae reach the lower epidermis. The end result was death and collapse of the host cells attacked and of the parasite.

A 2-day-old infection is shown in figure 2, *J*. The primary hypha at *b* is partly collapsed and the contents are dead. The host nucleus (*c*) shows a change in staining reaction. The chromatin material was positive to safranin rather than to the gentian-violet. This reaction is in line with that which occurred in the case of Yellow Transparent, although in Fameuse the death of the primary hypha seemed to be

more rapid. Intercellular penetration occurred at *a* and the secondary hypha has grown downward between two palisade cells but, as yet, has not produced a haustorium. The surrounding palisade and epidermal cells are unchanged.

Further contact with the host cells probably occurred soon after the second day. A 4-day-old infection (fig. 3, *F*) shows advancement of the hyphae into the second layer of palisade and spongy parenchyma tissues. Haustoria have entered several of the cells. The intercellular branch (*a* and *e*) has collapsed and most of the stainable contents now appear in the advancing hyphal tips at *d* and *g*. The palisade cell at *c*, probably the first in this region to be invaded, is dead. Other host cells are not seriously affected, although many of the haustoria are dead as shown at *f*. The primary hypha (*b*) is in a late stage of disintegration.

After the eighth day, development of the fungus ceased and the host cells and the mycelium degenerated rapidly. Analysis of the data showed that, although the fungus made only feeble growth during its existence, the actual death of the haustoria preceded serious injury to the host cells. A photomicrograph of a 15-day-old infection (pl. 1, *D*) shows that death and collapse of the tissues is nearly complete. No living hyphae or haustoria could be found. A group of cells from an 8-day-old infection (fig. 3, *A*) shows in detail the nature of the disintegration. At the margin of a 15-day-old lesion (fig. 4, *J*) dead haustoria occupy the broken-down protoplasts of their host cells at *a*, *b*, *d*, and *f* with invagination of the dead cytoplasm at *a*. In most cases the haustoria still keep their identity.

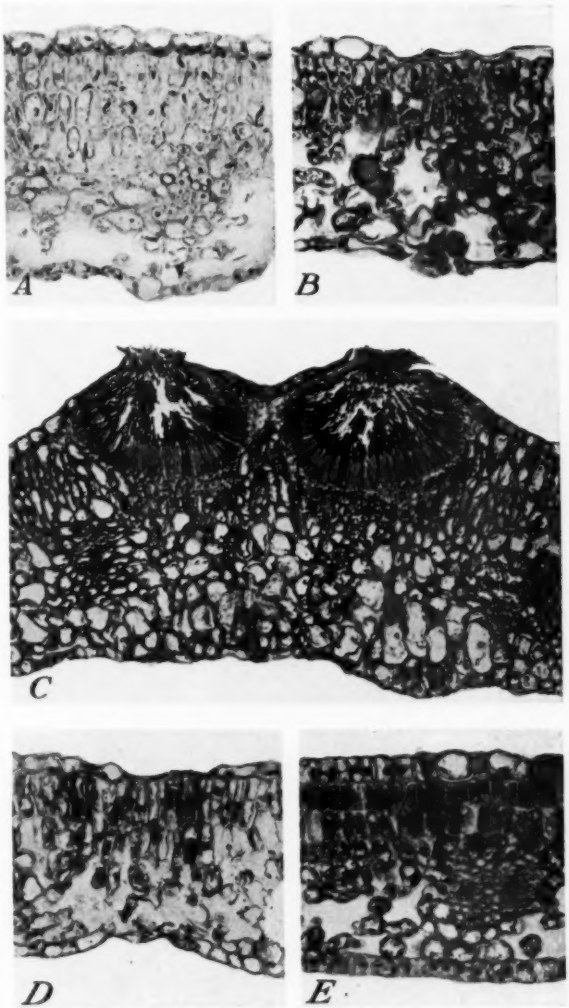
BALDWIN

The leaves of Baldwin were very resistant. The development of the fungus was restricted to the epidermal cell of entry and the adjacent palisade cells. Within 2 days after infection, the fungus died and shortly thereafter the adjoining host cells were violently affected.

A 2-day-old infection is shown in figure 2, *K*. An arrow indicates the point of entry. The primary hypha at *d* is dead. Intracellular penetration occurred and the secondary hypha (*e*) is also dead. The host nuclei at *a* and *c* within the parasitized cells are dead, and their state of collapse indicates that death followed soon after the fungus had succumbed. The host nucleus at *b* in the adjoining epidermal cell has enlarged.

A 4-day-old infection (fig. 3, *C*) shows the drastic effects upon the host cells in the lesion. The epidermal cell of entry has collapsed. It contains the dead remains of the primary hypha and of the dead host-cell protoplast. The intercellular secondary hypha is dead. A small haustorium at *a* died just as it entered a palisade cell. This cell is still alive and has just completed a division. Two palisade cells *b* are dead and a cell at *c* directly beneath these is disintegrating. All of the adjacent palisade cells contain faintly stained, vacuolated cytoplasm in contrast to the dense cell contents of the normal cells surrounding them. The plastids have disappeared and the nuclei have enlarged slightly. Here again the death of the fungus apparently preceded the collapse of the host cells and the reaction was more severe than in the case of Yellow Transparent or Fameuse.

A photomicrograph of a 3-day old infection (pl. 1, *E*) shows clearly the sharp differentiation between the dead fungus and the surrounding healthy host cells. The primary hypha and the intercellular hyphae



A, Nine-day-old infection on Wealthy, showing haustoria in many of the host cells, × 300. *B*, Fifteen-day-old lesion on Yellow Transparent, showing general collapse of host tissues in infected area, × 300. *C*, Fifteen-day-old infection on Wealthy, showing 2 pycnia and host reactions in their vicinity, × 150. *D*, Fifteen-day-old infection on Fameuse, showing collapse of palisade and upper epidermal regions in the infection, × 150. *E*, Three-day-old lesion on Baldwin, showing the dead fungus in contrast to the surrounding healthy host tissues, × 150.

in the underlying palisade appear as homogenous black strands in the photograph, indicating their total collapse. The epidermal cell of entry is degenerating and a palisade cell directly beneath, which was parasitized, is dead. Adjoining palisade and upper epidermal cells appear to be unaffected. These host tissues are clearly neither hypersensitive nor hypersusceptible. Starvation of the fungus also seems to be out of the question because the hyphae did not appear to be impoverished or depleted at the time of their collapse.

FURTHER OBSERVATIONS ON WEALTHY

DORSAL-SURFACE INFECTION ON YOUNG LEAVES

Successful infection of the lower surfaces of young Wealthy leaves was infrequent. Early stages in the development of such lesions were not encountered in the cytological preparations studied. About 12 days after inoculation, however, a few scattered rust spots became visible. Cytological study of these infections showed the character of host-parasite relations to be typical for the normal development in Wealthy leaves.

In all cases, penetration of the epidermal cells occurred freely and the primary hyphae expanded in the usual manner (fig. 4, *D*). The secondary branches (*b* and *c*) penetrated the large intercellular spaces of the spongy parenchyma and, therefore, failed to establish contact with the mesophyll cells of the leaf. Cessation of growth and death quickly followed. The case here described was typical of all of the younger infections, but the occupation of a single epidermal cell by more than one primary hypha was uncommon. None of the secondary hyphae were observed to emerge into adjoining epidermal cells.

INFECTION ON LEAVES PASSING INTO MATURITY

The types of reactions shown by Wealthy leaves which had just passed the period of expansion into full maturity are of special interest. Penetration occurred through the unwounded ventral epidermis and in 3 weeks the mycelium had spread approximately 225μ from the point of entry. Hypha ramified the mesophyll tissues to a considerable extent and nearly every host cell in the lesion was invaded by a haustorium. The host cells harboring haustoria became vacuolate and their cytoplasmic substances disappeared almost completely. Pycnia were not initiated. The group of cells from an 18-day-old infection (fig. 4, *G*) show almost complete cessation of fungal activity. Haustoria at *a* and *c* are functioning no longer and the hyphae at *d* and *e*, aside from their large nuclei, are nearly empty. The hypertrophy of host cells and collapse of their protoplasts common in older infections in younger leaves is not found here.

INFECTIONS ON OLD LEAVES

Penetration into the epidermal cells did not occur on the leaves of Wealthy which were approximately 3 weeks past the attainment of full size and maturity. This was true of both dorsal- and ventral-surface inoculations. The sporidia germinated in the usual manner with the production of short germ tubes, appressoria, and infection hyphae. An examination of several hundred cases showed that none of the infection hyphae was able to complete the penetration process. Many were found, however, where these minute pegs clearly penetrated the cuticle but failed to pass the epidermal cell walls. The

staining reactions in these preparations produced clear differentiation. The infection hyphae were dark violet in contrast to the transparent, light green of the cuticle and the dark, dull green of the epidermal cell walls. In figure 4, *A*, the infection hypha appears to be flattened against the cuticle. The application of pressure is quite apparent because the cuticle is slightly indented and the appressorium is raised and moved to one side. In *B*, the infection hypha has penetrated the cuticle and indented the epidermal cell wall. Another case *C* shows, in addition, the partial dislodgment of the appressorium. The type of resistance shown by these leaves seems to be of a purely physical nature.

DISCUSSION

The apple rust fungus is an obligate parasite and goes through its cycle of development only upon a living, susceptible host. When an apple leaf is exposed to infection under favorable external conditions, the paramount factors requisite to a successful infection, therefore, seem to fall naturally into three classes, as follows: (1) The ability of the sporidial germ tube to penetrate into the epidermal cell of the leaf, (2) the presence of a suitable substrate for the growth of the parasite in the cell protoplasts, and (3) the ability of the host cells to remain alive and function in a manner agreeable to the fungus. If this is true, any shortcomings in the production of a successful infection may be attributed to the absence of one or more of these requisites.

It has been shown that the apple rust fungus was able to penetrate the leaves of resistant varieties. No evidence was obtained which indicated that the parasite was excluded, even from the leaves of Baldwin, where no macroscopic signs of infection were detected. Gibson (*18*) observed that uredinial germ tubes entered the stomata of plants widely separated taxonomically, and concluded that penetration is not a reliable index of infecting capacity. This may have no application here, however, because the sporidial germ tubes of *Gymnosporangium juniperi-virginianae* penetrate directly. Melander and Craigie (*26*) found, in some cases, a correlation between resistance of the epidermis to puncture and rust resistance in the leaves of *Berberis*, the alternate host of *Puccinia graminis*. They found no evidence that the sporidial germ tubes entered an immune host like *B. thumbergii*, and concluded that morphological characters may account for resistance in some species. Although the observations made upon apple rust lend no support to the existence of varietal resistance due to morphological features, it is not impossible that such cases may be found in other varieties.

The immature leaves of Wealthy are susceptible to *G. juniperi-virginianae*. As long as the host cells remain alive they present a favorable medium for the development of the parasite. Dead haustoria are never found associated with living cells of this variety.

Varietal resistance appears to be due to some factor or factors in the host-cell protoplasts injurious to the fungus. This antagonism is severe in Baldwin, slightly less pronounced in Fameuse, mild in Yellow Transparent, and totally absent in Wealthy. The exact nature of the factor or factors involved still awaits solution and may be sought in an analysis of the chemical composition and physicochemical structure of the living host cells.

In lieu of an actual solution of the problem, various theories have been suggested. Many of these are based entirely upon microscopical evidence, but, recently, other methods of study have been employed. Ward (*34*) proposed that immunity was due to antagonistic reactions between host and parasite of the nature of the formation of toxins and antitoxins. Marryat (*25*), Gibson (*18*), Stakman (*33*), Allen (*1*), and others are generally in agreement with Ward. Leach (*22*) suggested a hypothesis based on a possible specific food requirement on the part of the fungus and the corresponding lack of this food substance within the host. Edgecombe (*15*) performed serological studies of wheats, susceptible and resistant to *Puccinia rubigo-vera tritici*. Purified globulin extracts of the grain were used as immunizing agents and the resulting antisera were given the precipitin test using the original extracts as test antigens. The results showed a relationship between precipitin reaction and rust resistance. Since the globulins used were believed to have been a part of the cytoplasm, it was reasoned that the genetic factors responsible for resistance were in some way associated with the factors producing the specific globulins. Leemann (*24*) proposed the theory that the holistic quality of the cell may play an important role in active plant immunity. It was based upon the assumption that the protoplasm of an immune host may not contain any specific substance deleterious to the invading organism, but that the living protoplasm as a whole, built up into a complex system, may present an unfavorable substrate. The phenol hypothesis of Newton and Anderson (*28*) suggests that rust resistance in wheat may be caused by the liberation of toxic phenolic compounds in the host cell upon the entrance of the fungus. This mechanism may explain different types of rust reactions when one considers the possible conditions following the action of fungal enzymes upon the phenolic compounds in the host-cell protoplasts.

Whether the antogonism against the apple rust fungus is already present in the make-up of the host-cell protoplasts or is induced by the parasite and presents a counterreaction against the intruder is open to question. Cytological evidence cannot settle this point. However, the substance or substances antagonistic to the fungus seem to be specific because many cases were found where the haustorium occupying the host cell had died and the cell itself showed no visible change. The reverse situation was found only in the older infections upon Wealthy and Yellow Transparent where the host apparently succumbed to the vigorous and prolonged attack by the parasite.

Regarding the interaction of host and parasite, many workers have observed peculiarities in the sequence of events. Ward (*34*), studying infections by *Puccinia dispersa* in resistant bromes, found that the germ tubes entered the stomata in a normal manner but either killed a few cells and then disintegrated or grew very slowly and failed to produce pustules. Marryat (*25*), working with *P. glumarum* upon the resistant American Club wheat, also noted irregularity. Stakman (*33*) observed injury of host cells to be a common phenomenon among plants resistant and immune to *P. graminis* and that the degree of hypersensitiveness varied in direct proportion to the degree of resistance. Regarding infection of *P. graminis tritici* upon the resistant Kanred wheat, Allen (*1, p. 148*) says, "* * * in one case the host cell is severely damaged at a time when the fungus is not visibly harmed, and in another the host cell is still normal in appearance

while the fungus has succumbed." According to Evans (*17*) no harmful effects to the host cells followed the invasion or establishment of the onion smut fungus, *Urocystis cepulae*, in either susceptible or nearly immune onion seedlings. In fact, mycelium which was badly degenerated or already dead was found in host cells which were still apparently healthy.

During active production of the pycnial exudate by the apple rust fungus, the mycelium seemed drained of its cytoplasm. Evans (*16*) and Allen (*2*), working with certain of the cereal rusts, noted a drainage of protoplasm from the central mycelium to the peripheral hyphae during fruiting. This does not seem to apply here because an accumulation of protoplasm in the peripheral hyphae is not found. A likely interpretation is that the contents of the hyphae are transported in some manner to the pycnia to support the prolific fruiting of the fungus. This is in agreement with the recent work of Liu [4] who made similar observations during his investigations of sexuality in *Gymnosporangium juniperi-virginianae*. The mechanism of this transfer is not understood, although it probably depends upon the presence of pores or other openings in the septa and a consistency of cytoplasm which would expedite movement. Allen (*2*) recognized this problem and suggested the possibility of some autolytic or digestive process reducing the hyphal contents to simpler soluble forms that would be more readily transportable. For a long time it has been known that protoplasmic streaming occurs in fungi, especially in the mucors and other groups having coenocytic hyphae. Buller (*13*) gives an excellent review of the literature on this subject. His own research showed that, in the higher fungi, pores were present in the septa through which protoplasm passed freely. Pores were not observed in the septa of the apple rust fungus. If, however, what Buller observed is applicable here, the passage of fungous protoplasm to a point where it is needed most may be explained.

It is also of interest that the marginal spread of the lesion almost ceased when pycnial formation began. Here again, it is believed that the fruiting activities of the fungus drew so heavily upon the food materials of the hyphae that energy for continued vegetative growth was not available.

Inoculations upon the dorsal surfaces of young Wealthy leaves usually failed. The secondary hyphae, emerging from the epidermal cells into the large intercellular spaces of the spongy parenchyma region, became shriveled and finally collapsed before they could establish contact with mesophyll cells. Death of the fungus by starvation seems to offer the most plausible explanation for this type of infection failure. In addition, the protoplasts of the lower epidermal cells may present a less suitable medium for the young parasite and the secondary hyphae possibly find the changing gas tensions of the intercellular spaces unfavorable during early development. The parasite certainly encounters a different environment in the lower epidermis as compared to the upper where 2 or 3 compact palisade layers without apparent intercellular spaces are present. It is, therefore, not surprising that the parasite behaves differently following lower-surface penetration.

[4] LIU, J. C. INVESTIGATIONS ON THE SEXUAL BEHAVIOR OF THE APPLE RUST FUNGUS. Unpublished doctoral dissertation. Univ. Wis. 1933.

The nature of resistance of mature Wealthy leaves is of special interest in view of the present lack of cytological evidence concerning this phenomenon.

Giddings (*19*) reported that York Imperial apple leaves were usually susceptible to rust for from 15 to 25 days after they unrolled from the buds, the length of the period being closely associated with growth conditions, and believed (*19, p. 33*)

* * * this acquired immunity in York Imperial leaves is due primarily to a change in materials available as food for the early nutrition of the fungus; and secondarily to certain changes in the thickness and composition of cell walls, leaf coverings, etc., which might be classed as physical.

Regarding this point, Reed and Crabill (*30*) also advanced the probability that the inhibiting factor was the increasing thickness of the cuticle and cell walls.

Giddings and Berg (*20*) found that mature leaves occasionally became infected through insect punctures and other wounds, but that infections of this kind developed very small lesions and aecia were never noted from them. This may lead to the belief that penetration was normally prevented but occurred when an avenue of entry was provided, and that, once inside the tissues, the fungus was unable to develop very far, due to some unfavorable situation in the mature leaf mesophyll.

The inoculation experiments of Miller (*27*) showed that sound, mature leaves of the susceptible varieties, Rome Beauty, Wealthy, and Jonathan, were resistant to apple rust infection but when the leaves were torn, prior to inoculation, lesions producing pycnia and aecia occurred along the edges of the wound. The mature leaves of the resistant varieties, Winesap and Delicious, showed no infection even when wounded. These data led to the interpretation that the resistance shown by mature leaves of susceptible varieties probably possessed a morphological basis.

In the present work, Wealthy leaves, which had reached full size at the time of inoculation, showed no visible infection. Microscopic examination, however, showed the presence of numerous lesions. Although actual penetration was not observed, no wounds were found near the point where entry was believed to have occurred. Some factor or factors prevented the fungus from following its normal cycle of development. A fundamental antagonism apparently does not exist. The evidence on this point is clear but interpretation is difficult and anything that might be said about it would be purely speculative.

The sporidial germ tubes failed to penetrate the epidermis of old Wealthy leaves. In some cases the infection hyphae punctured the cuticle, only to be stopped by the epidermal cell wall. In many instances, the cuticle and epidermal cell wall were indented and the appressorium partly dislodged from the leaf. These observations strongly suggest a physical ability of the epidermis to withstand the pressure of the penetrating infection hyphae.

SUMMARY

Cytological studies of apple rust infections in the leaves of four apple varieties, each differing from the other in its reaction to the rust fungus, are here reported.

The sporidial germ tubes penetrated the host directly and the process was essentially the same on each variety. The fungus began its parasitic activities intracellularly by developing a conspicuous primary hypha in an epidermal cell.

In young Wealthy leaves, the fungus developed vigorously for about 10 days after infection occurred. The uninucleate mycelium was regularly intercellular, sending characteristically uninucleate haustoria into the host cells. The haustoria were usually simple, rarely branched. Impoverishment of the parasitized host cells was slight, resulting generally only in a reduction in number of plastids.

Coincident with pycnial formation, marginal spread of the fungus almost ceased and the hyphae appeared to be partly depleted of their cytoplasmic contents. These phenomena are attributed to the fruiting activities of the fungus. It is thought that the food reserves in the hyphae, transported centripetally in some way to support the rapid exudation of the pycnia, were unavailable for further vegetative growth.

In the vicinity of the pycnia, the spongy parenchyma cells became hypertrophied and the protoplasts of the palisade and upper epidermal cells degenerated. The hypertrophy resulted in the obliteration of the large intercellular spaces in the spongy parenchyma region and the collapse of a portion of the lower epidermis. Haustoria occupying the broken-down cells were in various stages of collapse due to their unfavorable surrounding. As long as the host cells remained alive, however, they furnished a favorable substrate for fungal development.

In the leaves of the other varieties used, resistance was due to a distinct antagonism of the host-cell protoplasts to the invading parasite. The reaction was mild in Yellow Transparent, severe in Fameuse, and extremely severe in Baldwin. The failure of the fungus to establish itself could not be attributed to hypersensitiveness of the host cells, resulting in starvation of the obligate parasite. Death and collapse of the fungus preceded injury to the host cells.

Dorsal-surface infections upon young Wealthy leaves generally failed. Penetration occurred in the usual manner but the secondary hyphae, emerging from the epidermal cells of entry into the large intercellular spaces of the spongy parenchyma tissue, shriveled and collapsed before they could establish contact with mesophyll cells. This infection failure is attributed largely to starvation of the parasite.

As Wealthy leaves reached mature size, they became resistant. No macroscopic lesions appeared. The fungus, however, was able to penetrate and make considerable vegetative growth. Pycnia were not initiated. Aside from depletion of the invaded cells, the host showed little deleterious effects. Some situation, apparently, in the host, did not allow the fungus to follow its normal cycle of development.

The complete resistance shown by old Wealthy leaves is attributed to physical properties of the epidermis which successfully prevented the infection hyphae from gaining entrance into the host.

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INFLUENCE OF THE CARBOHYDRATE-NITROGEN RELATION ON NODULE PRODUCTION BY RED CLOVER[1]

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INTRODUCTION

There are many factors which may influence the production of nodules upon the roots of leguminous plants. Some of these are physical in character, such as light intensity, soil temperature, texture, and moisture; others are chemical in nature, such as soil acidity, inorganic salts, and simple or complex organic nitrogenous compounds. Of fundamental interest is one of a chemical character relating to the presence of certain inorganic salts in the substrate, viz, the quantity and kind of combined nitrogen.

The presence of nitrates and ammonium salts was known to reduce or completely suppress the number of nodules on inoculated legumes long before the experiments of Hellriegel and Wilfarth revealed the relationship between the centers of nitrogen fixation (the nodules) and their causal agents (the bacteria). The problem has attracted such wide interest that even to the present day researches have been conducted in the hope of determining the means by which the nodules are suppressed. A vast accumulation of data has resulted, but until recently attempts at interpretation have met with little success.

LITERATURE

During the 70 years that the inhibitory effect of inorganic nitrogen on legume nodulation has been recognized, many theories have been proposed to explain it, but the majority of these are supported by little or no experimental evidence. Briefly, the earlier theories were concerned with the toxic effect of nitrate nitrogen on the root-nodule bacteria, the production of a toxic principle in the plant, and immunity of the plant.

Mazé (*11*)[2] suggested that the combination of inorganic nitrogen with carbohydrate in the plant to form organic nitrogenous compounds effectively reduces the level of carbohydrate. As a result, excretion of carbohydrate from the roots is decreased and its chemotactic action on the bacteria reduced. Previous papers from this laboratory (*7*, *9*, *12*) have presented data which are readily explained by a modification of Mazé's theory; the modified hypothesis stresses the necessity of adequate carbohydrate supply in the interior of the plant rather than a hypothetical chemotactic effect. A paper of Fred and Wilson (*6*) summarizes the experimental results and the hypothesis offered in explanation.

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[2] Reference is made by number (italic) to Literature Cited, p. 611.

In field experiments, Rippel and Krause (*14*) show that the carbohydrate demands of the roots of the pea plant are much higher if nodules are present. The destruction of carbohydrate by the pea roots without nodules was similar to that of a nonleguminous plant. These observations are in complete agreement with the modified theory. Allison and Ludwig (*1*) reemphasize the importance of carbohydrate supply on nodule formation and suggest that the primary need of the carbohydrate is for tissue development rather than for energy purposes. They stress the increase in the top: root ratio with the addition of nitrogen, and affirm that nodule development is repressed by combined nitrogen for the same reason that root development is relatively decreased under the same treatment.

Hypotheses in explanation of this fundamental property of the fixation process in leguminous plants have been based for the most part on physiological experiments and have suffered from lack of experimental data of a biochemical nature. So far as the writer is aware, critical experiments to test the adequacy of a given hypothesis have not been made or even suggested by the various authors. Until these data are available, speculation is likely to replace conclusions based on experimental facts. This paper offers biochemical evidence bearing on the carbohydrate supply hypothesis gained from experiments of the following types:

(1) The carbohydrate supply with respect to the nitrogen was varied by regulating the pCO_2 [3] in the air that was supplied to red-clover plants, and the effect on the nodulation of the plants was studied.

(2) The level of carbohydrate in tops and roots of clover plants in which the carbohydrate-nitrogen relation varied was determined and correlated with invasion of the plant by bacteria and the development of the resulting nodules.

EXPERIMENTAL TECHNIQUE

CELLOPHANE CHAMBER

A suitable chamber for growth of plant cultures was devised, the details of which are shown in figure 1. The container is 20 inches high and 7 inches in diameter. It is covered with waterproof cellophane. The tin frame is sealed to a shallow porcelain pan by calking with plugging cotton and saturating the cotton with hot paraffin. A tube delivers the desired air plus CO_2 mixture to each pot after the mixture leaves the manifold. Details of the mechanics of mixing the gases for suitable atmospheres, filtering them, regulating their flow, and delivering them to the manifolds are given in a paper by Wilson and Georgi (*20*).

The growth of the plants in this type of container is as good as that of plants grown in open pots in the greenhouse. The chambers effectively keep out gross contamination without restricting growth or interfering with transpiration. Diffusion of CO_2 from a chamber is sufficiently slow so that concentration of this gas is higher than that of the surrounding air and can thus be readily maintained inside the container.

[3] pCO_2=partial pressure of CO_2 in atmosphere.

PLANT CULTURES

Plants of red clover (*Trifolium pratense*, var. Mammoth) were grown in sterilized half-gallon earthenware pots filled with a thin layer of gravel over which nitrogen-free pit sand was placed. A 25 by 200 mm glass tube in the center of the pot served as a means of watering; the surface of the sand was covered with sterile cork, which helped to prevent contamination by algae or micro-organisms.

The seeds were sterilized in Dakin's solution and germinated on sterile moist blotters in Petri dishes after the method of Hopkins, Wilson, and Fred (*8*). After the seeds had been allowed to germinate for 48 hours on the blotters, 10 to 12 seedlings were transferred aseptically to each pot. The seedlings were inoculated with a

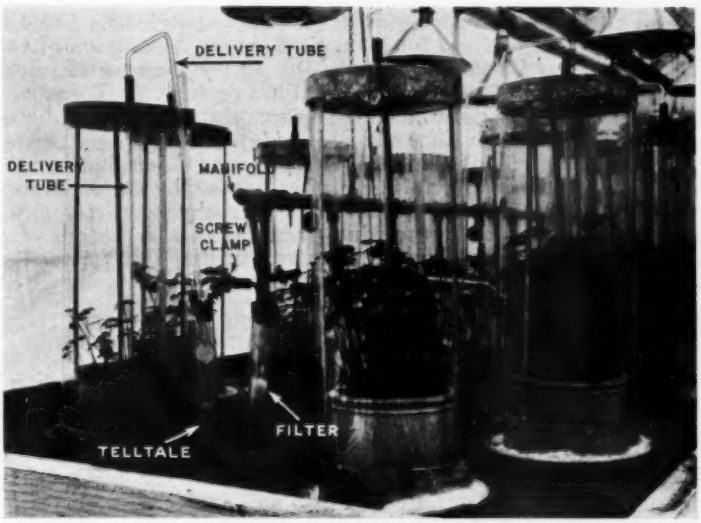

FIGURE 1.—Detail of cellophane chambers used for growing plant cultures.

water suspension of a 48-hour culture of *Rhizobium trifolii*. Strain 209, an effective one, was used. Loss of moisture from the pots was frequently checked, sterile water or nitrogen-free Crone's (*3*) solution being used to bring them up to weight. The nitrate nitrogen additions were made as sterile solutions in concentrations varying from 2.25 mg to 3.0 mg per cubic centimeter of solution.

Carbon-dioxide analyses of the atmospheres inside the cellophane chambers were made several times a week by methods developed in this laboratory (*16*, *19*, *21*). Atmospheres in the CO_2 series were found to vary from 0.1 to 0.25 percent. Sunlight was supplemented with artificial light during the fall, winter, and early spring months, as well as on cloudy days. The greenhouse temperature remained at about 25° C. throughout all of the experimental work.

HARVESTING AND CHEMICAL ANALYSES

The plants were washed from the pots at the time of harvest, special care being taken not to lose any of the nodules. After the nodules had been counted, the tissue was dried at 38° C. for 48 hours and the dry weight determined. The tissue was subsequently ground for analysis.

One-half of the plants used in experiments 5 to 7 were harvested 2 hours after sundown, at the close of the fifth or sixth week; the remainder, 10 days later. The roots were separated from the tops and the wet weight of each recorded. Both were then cut up into small bits, care being exercised to keep the loss of plant sap to a minimum. The samples for analysis were weighed as rapidly as possible to prevent the loss of moisture.

Total nitrogen determinations were made in experiments 1 to 4 on 0.75-g samples, as outlined for plant tissue in the Association of Official Agricultural Chemists' manual (*2*). In the presence of nitrate nitrogen the method recommended by Phillips et al. (*13*) was used. Nitrate nitrogen was determined on 1-g samples of dry tissue by the reduction method of Sessions and Shive (*15*).

In experiments 5 to 7, nitrate nitrogen was determined on 1-g portions of wet tissue immediately after harvest by the colorimetic method of Emmert (*4*). The total soluble carbohydrate fraction was analyzed according to the procedure recommended by Loomis et al. (*10*). Reducing sugars were determined by the micromethod of Stiles, Peterson, and Fred (*17*).

EFFECT OF INCREASED pCO_2 ON NODULE PRODUCTION BY CLOVER GROWN IN THE PRESENCE OF COMBINED NITROGEN

If the inhibiting effect of nitrate nitrogen on nodule production is correlated with the removal of the available total soluble carbohydrate by the inorganic nitrogen for protein synthesis, then the inhibition should be overcome if the sugar level in the plant sap is raised. Four experiments were made in which the pCO_2 in the atmosphere supplied inoculated clover plants furnished with various levels of combined nitrogen was increased in order to raise the carbohydrate content of the plant. Thus a critical test of the carbohydrate supply hypothesis could be obtained.

EXPERIMENT 1

These plants used in the first experiment were grown for 58 days in the fall of 1932. Ten milligrams of KNO_3 per eight plants per bottle were added at the start of the experiment and 10 milligrams weekly thereafter until the level of nitrate designated was reached. The first experiment was the only one not carried out in cellophane cans; instead, open 64-ounce flint glass bottles were employed, as described by Wilson and Georgi (*20*). Fifteen days after planting, CO_2 was started through the air plus CO_2 series.

The appearance of the tops (stems and leaves) was typical of the plants in subsequent experiments. The following details hold for all the experiments. Plants grown in a normal atmosphere were markedly smaller in height and leaf area than plants of the same NO_3 level enveloped in an atmosphere of increased pCO_2. The

color of the plants receiving nitrate nitrogen was a much deeper green than that of the controls (or 0-mg level), those exposed to the higher pCO_2 being a deeper green than those exposed to the normal pCO_2 of the air.

At the time of harvest the following observations were made with respect to nodule distribution. On the roots of plants of the 0-mg air series, nodules were fingerlike in shape and clustered around the crown of the taproot. In the 10-mg air series much the same picture was presented, but in the 20-mg air series nodules were small and round, indicating the "antagonistic" or inhibiting effect of the nitrate. In both the 40- and 80-mg air series, all of the nodules were small and round; at the latter level many plants possessed no nodules. In the air plus CO_2 series, a very different distribution took place. Plants at the 0-mg level possessed several very large fingerlike nodules on the crown of the taproot, and even the secondary roots contained nodules, both round and small fingerlike ones. At the 10-mg level, nodule distribution was very similar to that at the 0-mg level, except that more small, round nodules were in evidence. At the 20-mg level there were very few nodules on the taproot, the majority being relegated to the secondary root system. At both the 40- and 80-mg levels, the nodules were all on the secondary roots and small in size.

TABLE 1.—*Effect of increased pCO_2 on nodule production by red clover in the presence of KNO_3[1] in experiment 1 (58 days), Sept. 28 to Nov. 25, 1932*

Treatment		Plants				Nodules					
Atmosphere [2]	KNO_3	Total	Dry weight per 10—	Total nitrogen per 10 [3]—		Total count		Per grams (dry weight)		Per 100 milligrams of nitrogen	
	Milligrams	*Number*	*Grams*	*Milligrams*	*Percent*	*Number*	*Percent* [4]	*Number*	*Percent* [4]	*Number*	*Percent* [4]
Air	0	88	0.808	16.8	2.08	121±13.1	100	150	100	720.2	100
	10	56	.785	17.4	2.21	90± 4.4	74.4	115	76.7	517.2	71.8
	20	55	.724	16.0	2.21	63± 5.8	52.0	87	58.0	393.7	54.7
	40	60	.654	17.2	2.62	32± 4.2	26.5	49	32.7	186.1	25.8
	80	60	.725	19.7	2.71	3± 1.8	2.5	4.1	2.7	15.2	2.1
CO_2	0	83	1.476	24.7	1.68	145± 8.5	100	98	100	587.0	100
	10	59	1.015	21.7	2.14	111±11.0	76.6	109	111.2	511.5	87.1
	20	77	1.033	21.3	2.06	90± 8.2	62.1	87	88.8	422.9	72.0
	40	48	1.032	24.7	2.39	51± 3.9	35.2	49	50.0	206.5	35.2
	80	60	.908	20.2	2.22	33±10.2	22.8	36	36.7	163.4	27.8

[1] 10.0 mg added weekly to each pot; 10.0 mg added at start.
[2] CO_2 atmosphere contained approximately 0.15 percent CO_2.
[3] Nitrate nitrogen-free.
[4] Zero-mg level = 100 percent.

Table 1 gives the data pertinent to this experiment. There is marked evidence of the stimulatory effect of added CO_2 in both the total weight of nitrogen and in the average dry weight per 10 plants. The percentage of nitrogen is somewhat smaller in the air plus CO_2 series than in the air, indicating that the carbohydrate relative to nitrogen is higher in plants of this series. The total count of nodules per 10 plants is calculated in this table with the standard deviation.

A comparison of the total counts shows that plants receiving increased pCO_2 always possessed a greater number of nodules than the corresponding plants of the air atmosphere, i. e., plants re-

ceiving the same quantity of combined nitrogen. In all cases, the addition of NO_3 caused a reduction in the number of nodules, but the reduction was relatively less on the plants receiving additional CO_2. This is best brought out by comparison of the relative number of nodules on the plants of each NO_3 level with the 0-mg level equal to 100 in both the air and CO_2 series. In every case the relative number of nodules in corresponding levels of NO_3 was greater in the CO_2 series. This shows conclusively that the reduction was less in the CO_2 series.

In order to correct for the differences in size of plants, calculations were made on the number of nodules per gram of dry weight and per gram of nitrogen. Once again, comparing the relative values, it is seen that the reduction in the number of nodules (because of the presence of NO_3) is relatively smaller in the CO_2 series.

Attention is called to the fact that the absolute number of nodules per unit of weight or unit of nitrogen in the absence of nitrate nitrogen in the air series is greater, indicating that the centers of fixation (nodules) are more active on the plants receiving additional CO_2. This might arise from two factors: (1) Larger nodules, and hence a greater amount of tissue for fixation activity, or (2) the higher level of soluble carbohydrate that is probably available to the cells of the roots of the plants for tissue synthesis.

Under "total count", the stimulatory effect of the air plus CO_2 series (referred to from now on as the CO_2 series) over the air series is very marked, less so at the lower levels of nitrate. In the case of the highest level, 80 mg, the increase in the number of nodules of the CO_2 series over that of the air series is more than tenfold. The inhibitory effect of the nitrate nitrogen is evident within both the air and the CO_2 series, but it is to some extent overcome by an increase in pCO_2 furnished the plants. The same holds true for the number of nodules per gram of dry weight and the number per gram of nitrogen. The stimulatory effect of CO_2 calculated from the number of nodules per gram of nitrogen is marked, as can be seen by comparing the data shown for the two series in the last column of table 1.

EXPERIMENT 2

The plants in experiment 2 were harvested on the fifty-ninth day; they were grown during the winter months. The combined nitrogen was added as NH_4NO_3 to pots of 10 plants each. The following levels were used 0, 25, 50, and 100 mg per 10 plants. No nitrate was added to any of the plant cultures until the seventeenth day, when 25 mg was added to each of the NO_3 pots. Weekly thereafter, 25 mg was added to each of the 50- and 100-mg levels until the requisite quantity had been reached. Carbon dioxide was passed through the CO_2 series beginning with the seventeenth day of growth.

At the time of harvest the number of nodules within each series was found to decrease progressively with increasing nitrate nitrogen in all treatments; however, the number of nodules was greater in the CO_2 series. The plants in both 0-mg series had fingerlike nodules near the crown of the taproot, but those exposed to increased

pCO_2 possessed more and larger nodules, many of which were distributed over the secondary root system. The large fingerlike nodules found on the 0-mg CO_2 series were colored pink (anthocyanin pigments), characteristic of plant tissue exhibiting carbohydrate excess. In general, the effect of the NH_4NO_3 on nodule size and distribution was similar to that observed in experiment 1.

Figure 2 summarizes the essential data of experiment 2. At each of the nitrate levels the CO_2 series had almost twice as many nodules per 10 plants as the corresponding air series. A decrease in the total count within each series is evident with increasing nitrate nitrogen. Data not included in the graph showed that there was a marked increase in total nitrogen and dry weight in the CO_2 series, again almost twice that of the corresponding levels in the air series.

Figure 3 shows the characteristic distribution of nodules and the types of root systems of plants exposed and of plants not exposed to

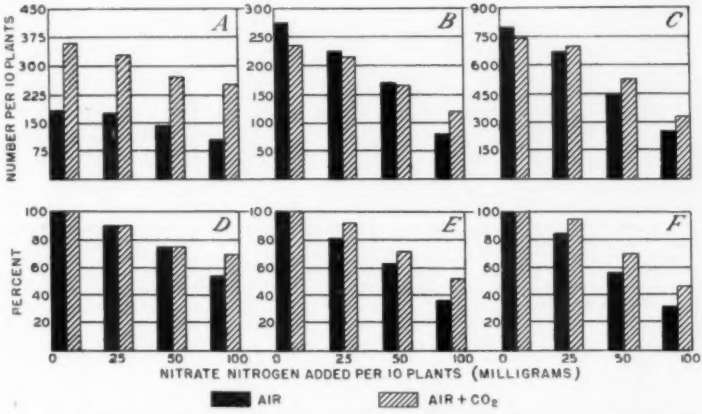

FIGURE 2.—Effect of increased pCO_2 on nodule production by red clover in the presence of NH_4NO_3, 25 mg being added to each pot at the start and 25 mg weekly; experiment 2 (59 days), November 20, 1932, to January 18, 1933. *A* and *D*, total count of nodules; *B* and *E*, nodules per gram of dry weight; *C* and *F*, nodules per gram of nitrogen. In *D*, *E*, and *F*, the 0-mg level = 100 percent.

an increased partial pressure of CO_2. In each part of the figure the 2 plants shown on the left were taken from the air series and the 2 on the right from the CO_2 series. The number, size, and distribution of nodules on the roots of the plants of the 2 different atmospheres and the 4-NO_3 levels are similar to those described previously and to those observed in subsequent experiments. It will be noted that the root systems are very much denser where the pCO_2 of the atmosphere surrounding the upper part of the plant is increased.

EXPERIMENT 3

The inorganic nitrogen source in experiment 3 was NH_4NO_3, all of which was added at two levels of nitrate nitrogen (50 and 100 mg per 10 plants per pot) at the start of the experiment. The plants were grown during the spring of the year and were harvested when they were 50 days old. CO_2 was first passed through the CO_2 series 4 weeks after planting.

The results of this experiment were very similar to those of the preceding experiments; that is, the number of nodules decreased with

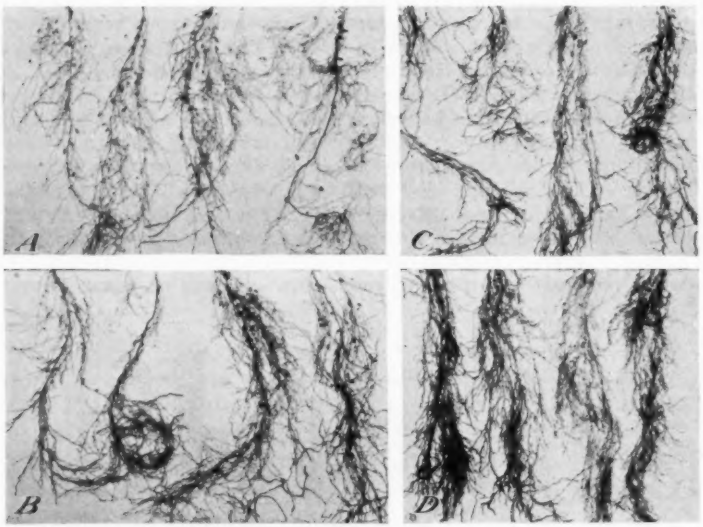

FIGURE 3.—Nodule distribution on roots of plants. These photographs are typical of plants of all four series. 0-mg level (*A*) and 25-mg level (*B*) are from experiment 2; 50-mg level (*C*) and 100-mg level (*D*) are from experiment 3. Note that the plants receiving increased pCO_2 present a striking degree of nodulation even though the nitrate nitrogen level has exceeded the critical concentration believed to be inhibitory under ordinary atmospheres. In each photograph plants grown in air are shown at left and those in air plus CO_2 at right.

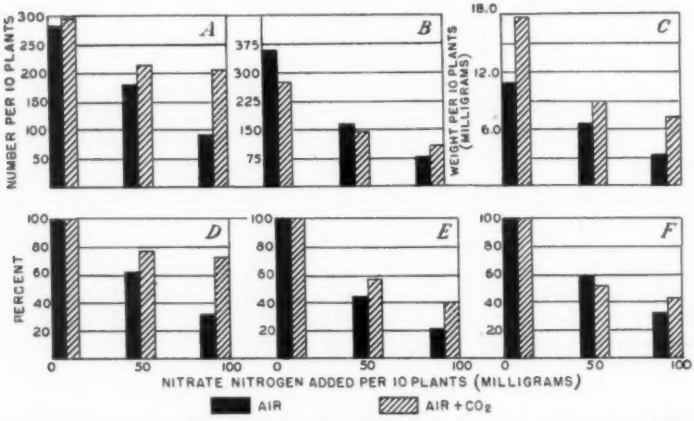

FIGURE 4.—Effect of increased pCO_2 on nodule production by red clover in the presence of NH_4NO_3, which was added to all pots at the start; experiment 3, (50 days) April 7 to May 27, 1933. *A* and *D*, total count of nodules; *B* and *E*, nodules per gram of dry weight; *C* and *F*, weight of nodules per 10 plants. In *D*, *E*, and *F* the 0-mg level=100 percent.

an increase in the NO_3 concentration and there was a markedly stimulating effect on nodule formation with increased pCO_2, with a

decided tendency for the nodules to be distributed over the secondary root system in the latter instance. Many of the plants at the 100-mg level in both series possessed no nodules, generally fewer being present on the roots of the 100-mg air series. In this experiment dry weight of nodule was determined; the data are summarized in figure 4. Both number and weight of nodule were greater on the plants given increased CO_2.

EXPERIMENT 4

Experiment 4 was conducted during the summer months for a period of 62 days. NH_4NO_3 at three levels—0, 50, and 100 mg—was employed; 25 mg were added at the start and 25 mg weekly thereafter. Passage of CO_2 was begun 21 days after the beginning of the experiment.

TABLE 2.—*Effect of increased pCO_2 on nodule production by red clover in the presence of NH_4NO_3,[1] in experiment 4 (62 days), July 5 to Sept. 5, 1933*

Treatment		Plants				Nodules					
Atmosphere [2]	NH_4NO_3	Total	Dry weight per 10	Total nitrogen per 10 [3]		Total count		Per gram (dry weight)		Per 100 milligrams of nitrogen	
	Milligrams	*Number*	*Grams*	*Milligrams*	*Percent*	*Number*	*Percent* [4]	*Number*	*Percent* [4]	*Number*	*Percent* [4]
Air	0	50	0.668	18.80	2.82	156	100	234	100	829.7	100
	50	65	.931	29.34	3.15	126	81	135	57.7	429.4	51.8
	100	70	.889	37.10	4.17	13	8.3	14.6	6.2	35.0	4.2
CO_2	0	50	1.024	27.82	2.72	164	100	160	100	589.5	100
	50	80	1.512	35.64	2.36	302	184	200	125	487.4	143.7
	100	84	1.845	54.25	2.94	92	56	50	31.2	169.6	28.8

[1] 25 mg. added at start; 25 mg. added weekly.
[2] CO_2 atmosphere contained approximately 0.15 percent CO_2.
[3] Nitrate nitrogen-free.
[4] 0-mg level=100-percent.

The nodule distribution was the same as in previous experiments. The complete data given in table 2 confirm the results of the other experiments in showing that with increased added combined nitrogen the percentage of nitrogen increased, the percentage of nitrogen was lower for a given treatment in the CO_2 series, and the inhibition of nodule formation both with respect to size and number was not so marked in the CO_2 series.

The plants of the 50-mg level of the CO_2 series were much larger than those of the 0-mg level of the same series. This stimulation of growth by the added combined nitrogen was accompanied by a marked increase in the number of nodules. It should be noted that with a similar increase in the size of plants (dry weight) in corresponding plants of the air series there was a decrease in the number of nodules.

EFFECT OF INCREASED pCO_2 ON SOLUBLE CARBOHYDRATE CONTENT OF INOCULATED CLOVER PLANTS FURNISHED COMBINED NITROGEN

The hypothesis, that the inhibitory effect of combined nitrogen on invasion of the plant and nodule development results from a decrease in the level of carbohydrate in the plant; is based on the assumption

that the combined nitrogen reduces this level by using it in protein synthesis. This assumption appears reasonable, but the necessary supporting data are meager. Strowd (*18*) and Orcutt and Wilson (*12*) have demonstrated that the addition of combined nitrogen reduces the carbohydrate content in the sap of the soybean as well as the number of nodules.

The explanation of the data obtained in experiments 1 to 4 would appear to rest on the increased carbohydrate level in the plant given added CO_2. Five experiments were made to determine the quantitative nature of this assumed increase; only three representative ones will be discussed at length.

EXPERIMENT 5

The plants used in experiment 5 were grown during the fall of 1933. The combined nitrogen was added weekly after the first 19 days of growth in the following order for the 50-mg level: 5, 10, 7.5,

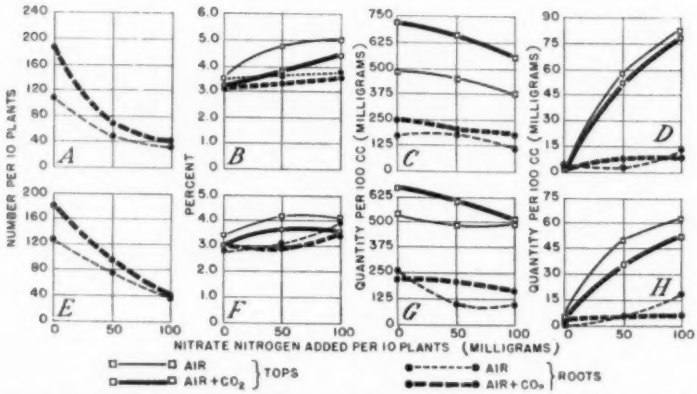

FIGURE 5.—Effect of increased pCO_2 on nodule production (*A* and *E*), percentage of nitrogen (*B* and *F*), invert sugar in tops and roots (*C* and *G*), and inorganic (NO_3) nitrogen (*D* and *H*) in red clover grown in the presence of Ca $(NO_3)_2$ in experiment 5; first harvest is shown in *A* to *D* and second harvest in *E* to *H*.

10, and 17.5 mg of $Ca(NO_3)_2$, the last amount being added on the forty-second day of growth, or 13 days before the first harvest. For the 100-mg level, there were added weekly after the first 19 days, 10, 20, 15, 20, and 35 mg per 10 plants per pot.

At the first harvest, the clover plants were 55 days old; at the second, 65 days old. CO_2 was first passed through when the plants were 21 days old, those of harvest 1, CO_2 series, being exposed to CO_2 for 34 days and those of harvest 2 for 44 days.

The inhibitory effect of $Ca(NO_3)_2$ on nodule production with the partial effectiveness of CO_2 is demonstrated in figure 5, *A* and *E*. The percentage of nitrogen is lower, generally, in the CO_2 series than in the air series; with increasing nitrate nitrogen, the percentage of nitrogen likewise increased in both series.

With increasing nitrate nitrogen concentration, the level of soluble carbohydrate tended to fall off in both roots and tops. A higher level of sugar in the sap of plants grown in increased pCO_2 is strik-

ingly illustrated. The nitrate nitrogen concentration within the plant increased but not proportionately to the quantity added to the substrate. Here the plants of the CO_2 series showed less nitrate nitrogen in the plant sap than those of the air.

EXPERIMENT 6

Experiment 6 was conducted in the spring. Half of the $Ca(NO_3)_2$ of the two nitrate levels was added at the time of planting, the rest, i. e., 25 and 50 mg, was added to the 50- and 100-mg levels, respectively, 23 days later, thereby making all of the combined nitrogen present 31 days before the first harvest.

At 54 days, harvest 1 was made, and at 61 days, harvest 2; CO_2 was started 23 days after planting.

The data in this experiment, given in figure 6, bear a marked similarity to those of experiment 5 (fig. 5). There is a tendency

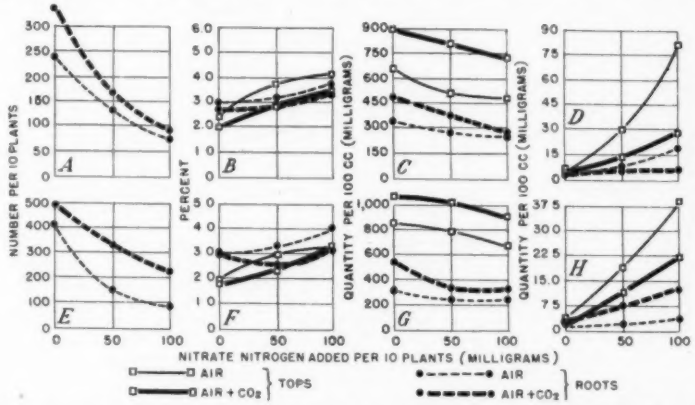

FIGURE 6.—Effect of increased pCO_2 on nodule production (*A* and *E*), percentage of nitrogen (*B* and *F*), invert sugar in tops and roots (*C* and *G*), and inorganic (NO_3) nitrogen (*D* and *H*) in red clover grown in the presence of Ca $(NO_3)_2$ in experiment 6; first harvest is shown in *A* to *D* and second harvest in *E* to *H*.

for the number of nodules to decrease at increasing levels of nitrate nitrogen, and again the CO_2 series maintains a higher margin than does the air series at the three NO_3 levels. This is more marked in the second harvest than in the first. The percentage of nitrogen increases with added combined nitrogen and is always higher in the air series for a given level of combined nitrogen. The invert sugar decreases with increasing NO_3 concentration, again a higher level being maintained by the CO_2 series.

The nitrate nitrogen in the plant sap increased with the concentration, as would be expected, analysis of the CO_2 series indicating that less existed there than in the air series. This increase was much more marked in the top portions of the plant in this experiment than in those of experiment 5. For some unknown reason, in harvest 2, the CO_2 roots possessed a greater quantity of nitrate nitrogen than the corresponding air series. This is the only instance in which such a discrepancy occurred.

EXPERIMENT 7

Because of the limited number of cellophane cans, it was necessary to employ fourteen 12-l pyrex bottles in order to carry on this experiment simultaneously with the preceding one. The technique was varied somewhat in that 20 seeds were planted per bottle and consequently the nitrate nitrogen level had to be raised in order to have nitrate present in the same proportion that it was when 10 plants per pot were used. The levels chosen were 100 and 200 mg per 20 plants per bottle. The system of growth employed was similar to that described in a previous publication (*20*) for 64-ounce glass bottles. Carbon dioxide determinations were made colorimetrically in small flasks suspended permanently in each bottle (*16*).

The experiment was carried on in the spring. The $Ca(NO_3)_2$ was added in two portions, one-half at the time of planting and the remainder at the close of 22 days.

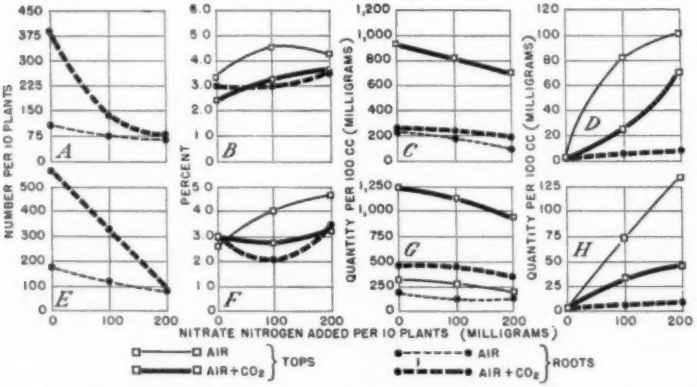

FIGURE 7.—Effect of increased pCO_2 on nodule production (*A* and *E*), percentage of nitrogen (*B* and *F*), invert sugar in tops and roots (*C* and *G*), and inorganic (NO_3) nitrogen (*D* and *H*) in red clover grown in the presence of $Ca(NO_3)_2$ in experiment 7; first harvest is shown in *A* to *D* and second harvest in *E* to *H*.

Harvest 1 was made when the plants were 43 days old and harvest 2 a week later. CO_2 was passed through after 22 days of growth.

The data are presented in figure 7. The general tendency is very similar to that of the preceding two experiments, except that the soluble sugars of the roots of the CO_2 series of both harvests exceed those of the tops of the air series of the same harvests. This is believed to have been caused by the lack of CO_2 in the air series. As these plants grew larger, it was found increasingly difficult to supply them with the requisite quantity of CO_2 because of the rapid assimilation, even though adjustments were frequently made to supply them at increased rates of gas flow.

The data are not complete because of the lack of root tissue in the air series of both harvests, hence there are no curves for the air roots in the percentage nitrogen or nitrate nitrogen determinations; all of the tissue was used for the carbohydrate determination and even that proved insufficient for the first harvest. The number of nodules de-

creased with increasing nitrate nitrogen concentration. This was especially true of the CO_2 series, which is contrary to the results of previous experiments. However, the nodules on the plants given increased CO_2 were much larger than those in the corresponding series given air. The percentage of nitrogen again is higher in the air series than in the CO_2 series. The soluble sugars decreased as in previous experiments, the CO_2 series maintaining a higher level in the sap of both tops and roots. The nitrate nitrogen in the sap increased with an increase of NO_3 in the substrate, the air series indicating much more present than the CO_2 series.

DISCUSSION

The quantitative data presented in this paper substitute experimental evidence for some of the assumptions on which is based the hypothesis that inorganic nitrogen reduces nodule formation in leguminous plants by decreasing the carbohydrate supply. Before discussing the interpretation of the data in relation to this hypothesis, it may be well to summarize the salient facts of these experiments.

(1) If an inoculated clover plant is furnished combined nitrogen, the number and size of the nodules decrease; with increasing quantities of nitrogen, the development of the nodules is inhibited to the vanishing point.

(2) Coincident with this antagonistic effect of combined nitrogen on nodule formation may be noted: (*a*) an increase in the percentage of nitrogen (narrowing of the carbohydrate-nitrogen relation in the plant); (*b*) a decrease in the quantity of soluble sugars in the sap; (*c*) an increase in the inorganic (nitrate) nitrogen.

If plants receiving combined nitrogen in quantities sufficiently large to inhibit or to suppress nodule formation are furnished an atmosphere containing increased CO_2, the foregoing responses are modified considerably.

(3) The invasion of the plant and the development of the nodules are greatly stimulated by increased CO_2 so that the inhibitory effects of the combined nitrogen can be partially but not completely overcome. This beneficial effect on nodule formation may be demonstrated by using the following criteria of nodule production either on an absolute or on a relative basis: (*a*) Number of nodules per plant, (*b*) number of nodules per gram dry weight of plant, (*c*) number of nodules per gram nitrogen of plant, (*d*) dry weight of nodules per plant.

(4) Accompanying the beneficial effect of increased CO_2 on nodule formation in the presence of combined nitrogen are: (*a*) A decrease in the percentage of nitrogen in the plants indicative of a widening of the carbohydrate-nitrogen relation obtaining therein; (*b*) an increase in the total soluble sugars in the sap, i. e., for a given level of combined nitrogen nutrition, plants supplied with increased CO_2 will have a higher content of carbohydrate; (*c*) a decrease in the level of inorganic nitrogen in the plant.

These results supply the needed experimental basis for the carbohydrate-supply hypothesis, and in a general way substantiate this explanation of the combined nitrogen effect. Qualitatively, the data show that if combined nitrogen is supplied to inoculated leguminous plants, the level of a soluble carbohydrate and the C:N relation decrease coincidently with a decrease in nodule development. If the level of carbohydrate and the C:N relation are kept from changing in the presence of added combined nitrogen by increasing the plant's photosynthetic activities, the inhibitory effect of the combined nitrogen can be partially overcome. It seems reasonable to suppose, then, that there is an intimate interrelationship between the carbohydrate supply, the nitrogen nutrition, and the formation of nodules.

Quantitatively, the data suggest that this interrelationship is not the sole factor that conditions the response of the nodules to the presence of combined nitrogen. This is brought out in experiments

5 to 7. Although, as figures 5 to 7 show, the presence of combined nitrogen results in a decrease in the soluble sugar content of the sap, the observed decrease does not appear to be sufficiently large to account for the striking reduction in the number and size of the nodules. It is realized that the figures for the individual determinations are subject to daily and even hourly variations; hence, conclusions must be based on a fairly large number of samples. The numerous analyses made in this study as well as those on soybeans presented by Orcutt and Wilson (*12*) consistently show that although a definite reduction in sugar takes place in plants furnished combined nitrogen, the observed decrease does not appear to be sufficient to account completely for the accompanying reduction in number and size of the nodules.

In view of the relatively greater effect of combined nitrogen in decreasing development of nodule tissue than in decreasing the carbohydrate level in the plant sap, an explanation of the reduction of nodules by nitrates based solely on decrease in soluble carbohydrate appears inadequate. The following alternative explanations are available:

(1) The carbohydrate level in the plants, especially in the roots, is in equilibrium with the requirements of host and bacteria, and any change in the available supply results in a decrease below which normal growth cannot occur; a small decrease in the available carbohydrate may result in a large decrease in the development of nodular tissue. This view has been stressed by Allison and Ludwig (*1*), but no data are available that directly support the assumption. Until more definite information is obtained, judgment with respect to the adequacy of this explanation must be reserved.

(2) A second explanation that might be suggested is that the soluble carbohydrate figures are only a rough indication of the total supply of energy and building material, and that although nitrate reduces this supply quite markedly, there is a tendency for the soluble fractions to remain more or less constant. Lacking definite information with respect to which types of carbohydrates are important in this connection, the required analyses would have to include all forms—a laborious proceeding. This idea, however, can be tested in a general way by regarding the percentage of nitrogen in the plant as an inverse measure of total carbohydrate and determining whether or not plants given both CO_2 and combined nitrogen respond with respect to total carbohydrate and nodule production as do plants grown in air. To decide this question, the data from 10 experiments were combined and the correlation coefficients between number of nodules and percentage of nitrogen were determined.

TABLE 3.—*Correlation coefficients between number of nodules and percentage of nitrogen*

Item	r	z [1]	Probability [2]	$n-3$	Reciprocal	Remarks
CO_2	−0.6896	−0.8472	<0.01	45	0.0222	Definite negative correlation in both series; difference not significant.
Air	−.6666	−.7940	<.01	42	.0237	
Difference		0.0532±0.214		Sum	0.0459	

[1] For z test see Fisher (*5*, ch. 6–7).
[2] Probability that observed correlation arose through random sampling.

The coefficients, given in table 3, show that there is a decidedly significant inverse correlation between percentage of nitrogen and number of nodules, or a direct correlation between total carbohydrate and number of nodules. Moreover, the difference between the plants supplied with increased CO_2 and those given normal air is not significant, indicating that these two populations belong together. It is therefore concluded that, insofar as these data can supply an answer, nodule production varies directly with total carbohydrates, and the response is consistent irrespective of the treatment. It is realized that this conclusion cannot be regarded as final, since both variables (total carbohydrates and nodules) were crudely measured; possibly data concerned with various forms of carbohydrate and with weight or volume of nodules will supply a definite answer.

(3) Finally, the data are not inconsistent with the view that there is a specific effect of the combined nitrogen ion. Reference to figures 5, 6, and 7 shows that coincident with the increase in soluble carbohydrate and number of nodules, there occurred a decrease in the concentration of the NO_3 in the sap of plants given additional CO_2. It is possible that the NO_3 exerts a specific effect in addition to or independent of its indirect effect in lowering available carbohydrate.

SUMMARY

The inhibitional effects of combined nitrogen upon nodule production by inoculated red clover plants can be partially overcome by increasing the carbohydrate synthesis in the plant; that is, by supplying inoculated plants given combined nitrogen with additional CO_2.

Coincident with overcoming this inhibition there occurs: (1) a rise in concentration of the soluble carbohydrate in the sap, (2) a decrease in the percentage of nitrogen (widening of the C:N relation), and (3) a decrease in the concentration of combined inorganic nitrogen in the sap.

These responses are discussed with reference to their bearing on the theory that the inhibitional effects of combined nitrogen depend on the influence of the latter on available carbohydrate.

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THE RELATION OF FOLIAGE COLOR TO APHID RESISTANCE IN SOME VARIETIES OF CANNING PEAS[1]

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INTRODUCTION

Foliage color of canning peas varies between varieties, and occasionally within varieties, from a deep green to a light green which closely approaches yellow. As the writer has pointed out,[2] certain varieties of canning peas (*Pisum sativum* L.) vary widely in the degree to which they are damaged by the pea aphid (*Illinoia pisi* (Kalt.)) during seasons of severe infestation. In such years a constant relation has been observed between color of foliage and the reaction of the plant to insect injury, resistance being associated with yellow in the foliage and susceptibility with green. This paper reports the results of a study made to obtain evidence concerning this apparent relation between resistance and foliage color. A number of crosses were made between yellow- and green-foliaged varieties and the progenies studied. In this paper plants with green foliage are referred to as "green" and those with yellow foliage as "yellow."

MATERIALS

Three varieties of canning peas, Yellow Admiral, Perfection, and Onward, were used in this study. Yellow Admiral was selected because of its distinctly yellowish foliage and its observed resistance to aphid attack. This variety has a tall vine with long, slender internodes and small leaves. Perfection, which is very susceptible, is a deep-green dwarf pea with short, thick internodes and large, fleshy leaves. Onward resembles Yellow Admiral in foliage color and resistance, and Perfection in habit of growth. In 1931 these three varieties were hybridized with each other, making all possible combinations. The first generation of each cross was grown in the greenhouse during the winter of 1931–32. The second generation was grown, with woven-wire fences as supports, in the field in 1932. The entire population from each cross was grown on a separate fence, and the individuals were evaluated whenever possible as to height and color. The F_2 progeny of the Yellow Admiral-Perfection cross was separated into three classes—tall green, tall yellow, and dwarf green. The F_2 progeny of the Yellow Admiral-Onward cross also contained both tall and dwarf plants that were distinctly green, although the foliage of both parents was yellow. Therefore four classes were separated from the F_2 population of this cross—tall green, tall yellow, dwarf green, and dwarf yellow. Two classes were selected from the Onward-Perfection cross—dwarf green and dwarf yellow. Seed saved from individual plants in each class was planted in 1932 for F_3 progeny tests.

[1] Received for publication May 8, 1935; issued December 1935.

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It was not possible to determine the number of individuals in each class from each cross in the F_2 population. Many of the plants became infested with a virus disease which caused a chlorosis of the plants and rendered normally green plants quite indistinguishable from yellow ones.

In a subsequent cross between Yellow Admiral and Onward it was found that in the F_2 population of 164 individuals, 90 were tall green, 33 were tall yellow, 26 were dwarf green, and 15 were dwarf yellow. Dwarf is a simple recessive to tall, but the fact that yellow and green are distributed in approximately a 3:1 ratio in each of the height classes may be merely a coincidence. Green plants predominated in a cross involving two yellow strains, thereby indicating that foliage color may rest upon a complex genetic basis. Somewhat arbitrary distinctions as to color value are necessary in classifying an entire population in this way since there are at present no satisfactory methods of evaluating foliage color under field conditions. Many plants were found the color of which was intermediate between the colors of the parents. These were not used since they could not be assigned either definite or comparative values.

F_4 progenies were not used in aphid-resistance tests but were grown for increase and further elimination of lines segregating for vine height and foliage color. The F_5 progeny test was made in 1933 upon dwarf plants which had been carefully selected in the F_4 for uniformity of height and color.

PROCEDURE

In testing the F_3 populations each class from each cross was planted in a solid block. When the plants were about 3 inches high they were dusted with nicotine dust to remove any aphids that might be present and were then given a uniform aphid infestation. The aphids used for this purpose were reared on a susceptible strain of peas grown in greenhouse flats. These aphids had been brushed from the plants to the soil periodically. In crawling back to the plants they tended to distribute themselves uniformly among the plants. Aphid-laden plants were cut off at the ground and methodically placed upon the test plants.

When the plants were in full bloom a series of four aphid counts was made. This was done by bending the top 5 inches of each of 10 plants over a metal container, knocking the aphids off into the container, and counting them. At the time the counts were made many tall and green families of the F_3 were still producing dwarf and yellow plants. For this reason, only plants typical of the class in which they grew were selected for aphid counts.

Only dwarf families were used in the F_5 test. Each family was planted in 3 rows 5 feet long, 8 inches apart, and 18 inches from the next nearest family. The families were planted on August 1, 1933, in 3 groups of 14 families to each. Each group extended east and west and each family extended north and south. Plantings of parent Perfection stock were made at irregular intervals to serve as checks. The groups were lettered alphabetically, beginning at the north, while the families were numbered 1 to 14 beginning at the east. Families 1 and 14 were border families and were not used. These families were infested as heretofore except that aphids were removed from the susceptible plants by means of a small camel's-hair brush

and placed upon the leaves of the test plants. Sixteen aphids of all ages were thus placed upon each row at regular intervals.

Four aphid counts were made at 2-day intervals after the plants were in full bloom. In order to standardize the procedure the five plants growing at the south end of the eastern row of each family were cut off at the ground, all aphids were shaken from the plant into a metal container, and the aphids counted. The next five plants were taken for each succeeding count.

EXPERIMENTAL RESULTS

TABLE 1.—*Number of aphids occurring on green as compared with yellow segregates in third-generation selections, Madison, Wis., 1932*

Cross	Type of F_3 segregate	Infestation per 10 F_3 plants			
		Sept. 22	Sept. 24	Sept. 26	Sept. 28
Perfection × Yellow Admiral	Tall green	644	760	1,360	1,933
	Tall yellow	121	107	144	196
	Dwarf green	634	358	1,936	2,800
Onward × Yellow Admiral	Tall green	1,840	970	1,384	2,024
	Tall yellow	97	366	294	288
	Dwarf yellow	282	668	776	1,512
Perfection × Onward	Dwarf green	5,746	5,040	7,680	10,524
	Dwarf yellow	1,258	688	720	2,080

In the results of the F_3 test, given in table 1, the classes are arranged in couplets. Thus in the Yellow Admiral × Perfection hybrids the tall green class differs from the tall yellow class chiefly in color.

TABLE 2.—*Number of aphids per five plants occurring on green as compared with yellow fifth-generation families from individual plant selections, and on Perfection parent stock which was used as a check, Madison, Wis., 1933*

Green segregates					Yellow segregates					Perfection checks				
Family	Sept. 12	Sept. 14	Sept. 16	Sept. 18	Family	Sept. 12	Sept. 14	Sept. 16	Sept. 18	Family	Sept. 12	Sept. 14	Sept. 16	Sept. 18
A2	1,408	1,872	7,497	10,166	A3	1,552	2,412	5,296	(1)	A5	3,200	5,089	9,863	(1)
A4	2,610	4,770	11,783	(1)	A6	1,576	2,064	6,928	(1)	A9	6,400	9,281	14,981	(1)
A7	1,774	3,072	10,630	(1)	A10	1,400	2,191	6,986	(1)	A13	1,464	2,769	12,550	(1)
A8	2,208	4,353	3,650	(1)	A12	1,784	3,436	3,777	(1)	B3	2,624	4,770	6,659	(1)
A11	2,060	2,143	6,179	(1)	B4	1,606	2,266	6,872	10,377	B6	4,320	----------	10,051	(1)
B2	476	1,120	6,783	16,393	B5	520	896	3,225	8,065	B10	1,824	4,385	9,733	(1)
B7	1,104	5,394	9,153	14,730	B12	1,320	1,224	3,104	8,194	C8	1,440	----------	5,667	7,870
B8	1,456	1,823	7,042	14,600	C3	520	2,138	5,413	7,620					
B9	580	2,305	7,821	(1)	C7	260	928	1,725	4,090	Total	21,272	26,294	69,504	7,870
B11	1,552	2,031	4,352	5,563	C10	788	2,097	3,714	3,200					
B13	756	936	4,578	8,769	C13	624	524	1,125	2,944	Mean	3,038.9	5,258.8	9,929.1	----------
C2	712	1,429	1,944	10,120						Mean gain	----------	2,219.9	4,670.3	----------
C4	1,986	1,736	3,135	5,953	Total	11,950	20,176	48,165	44,490					
C5	1,040	1,440	4,255	10,440										
C6	792	1,680	4,288	11,140	Mean	1,086.4	1,834.2	4,378.6	6,355.7					
C9	840	1,208	6,178	7,490	Mean gain	----------	747.8	2,544.4	1,977.1					
C11	1,296	2,657	4,516	6,010										
C12	488	1,456	4,418	5,600										
Total	23,158	41,425	108,202	126,974										
Mean	1,286.6	2,301.4	6,011.2	9,767.2										
Mean gain	----------	1,014.8	3,709.8	3,756.0										
Difference [2]	200.2	467.2	1,632.6	3,411.5										

[1] Dead.
[2] Cumulative difference, greens over yellows.

As previously stated, resistance tests were not made with F_4 progenies, but F_5 progenies which appeared to be fixed for color and vine height were subjected to aphid infestation in 1933. In table 2 the infestations of the yellow progenies, the green progenies, and the Perfection check rows are listed separately, also.

In figure 1 the comparative rate of increase and number of aphids per five plants on yellow and green plants are shown. A statistical treatment of the data is given in table 3.

The data show that in all cases the green plants were more heavily infested than the comparable yellow plants. Thus in table 1 the

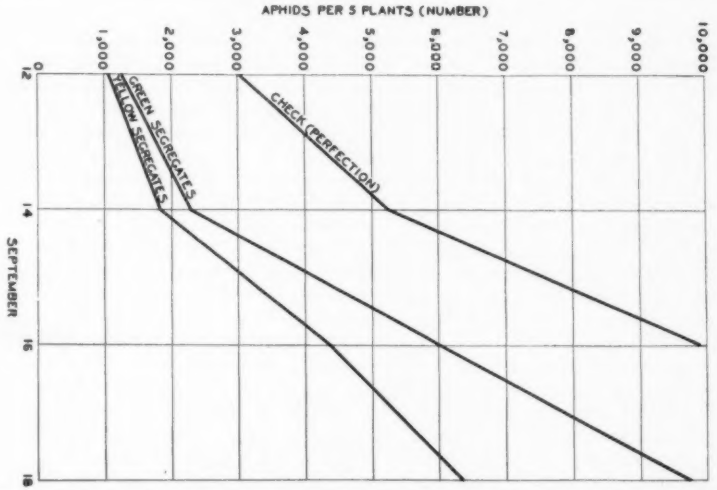

FIGURE 1.—Comparative rates of increase in aphid population on green and yellow segregates and on the green variety used as a check, Madison, Wis., 1933.

tall green class from the Yellow Admiral × Perfection cross had about 10 times as many aphids on September 28 as the tall yellow plants. A comparable difference was observed between the tall green and the tall yellow classes from the Yellow Admiral × Onward cross. The only direct comparison possible between dwarf green and dwarf yellow classes is in the progenies from the Perfection × Onward cross. Here there were about 5 times as many aphids on the dwarf green plants as on the yellow at the end of the experiment. It should be noted again that only plants at the obvious extreme of either green or yellow were used in this study.

The third-generation results show clearly that resistance to aphids is heritable and is associated closely with the expression of yellow-colored foliage. Green progenies from a cross between Yellow Admiral and Onward, two yellow varieties, were as susceptible as any comparable green varieties, while the yellow progenies were resistant.

TABLE 3.—*Statistical treatment of data in table 2 showing means, standard deviation, difference between means, standard error of the difference, and ratio of latter two to each other, Madison, Wis., 1933*

Date	Green		Yellow		Mean of green minus mean of yellow	Standard error	Critical ratio
	Mean	Standard deviation	Mean	Standard deviation			
Sept. 12	1,286.6	642.4	1,086.4	547.1	200.2	223.9	.....
Sept. 14	2,301.4	1,294.3	1,834.2	849.6	467.2	398.4	1.17
Sept. 16	6,011.2	2,631.7	4,378.6	2,069.1	1,632.6	879.8	1.86
Sept. 18	9,767.2	3,701.3	6,355.7	2,909.8	3,411.5	1,504.4	2.27

There was considerable variation in the number of aphids which developed upon the different progenies within each class, as shown in table 2. In every case, however, the mean infestation on the green class was higher than the mean on the comparable yellow class. The acceleration of aphid numbers was also much greater on the former than on the latter (fig. 1).

Figure 1 sums up the differences in resistance between plants of the two foliage colors. All of the families dealt with mature at about the same time and, in the absence of aphids, produce satisfactory yields of peas. Owing to the absence of some factor in the green peas or the presence of some factor in the yellow peas, or vice versa, the aphids increased to lethal numbers on the green peas before they did in the yellow peas. These data indicate that in this instance the intensity of aphid population at which well developed plants were permanently wilted was about 3,000 aphids per plant. Eventually the yellow peas were destroyed also by an aphid population which appeared about as intense as that at which the green plants were destroyed, although no counts were made after September 18. This would indicate that the number of aphids which cause permanent wilting is about the same for both classes and that actual resistance rather than tolerance or avoidance is the cause of the slower acceleration of aphid numbers upon the yellow plants. The practical difference in resistance between the two hybrid groups worked with might be expressed in this difference in time in which the two groups are destroyed. If there were so few aphids that the green peas could produce a satisfactory crop before being destroyed there would be no practical difference in the expression of resistance. If the infestation were so severe that the yellow plants were also destroyed before producing satisfactorily, there would again be no practical difference. When the intensity of aphid population is such that the green peas are destroyed while yellow peas produce a satisfactory crop under comparable conditions because of the slower acceleration of aphid numbers, then there is a practical difference which may be expressed either in terms of relative acceleration of aphid numbers or in days elapsed between dates on which the critical intensity of population is reached on the different groups. This difference may be attributed to some factor for resistance in the yellow varieties. Figure 1 shows this difference. The acceleration of aphid numbers on the yellow plants was so much slower than on the green plants that many of the yellow families produced peas of canning size before they were destroyed, whereas none of the green families produced any usable peas. All of the green families had been destroyed

within a few days after the last infestation was determined. The yellow segregates lived several days longer. It is plainly evident that, with the same initial infestation, the aphids reached destructive numbers on the green plants more quickly than on the yellow plants.

Figure 1 also shows the acceleration of aphid numbers upon the check plants. Here it is quite obvious that the parent stock was even more susceptible than the segregates with apparently similarly colored foliage. The difference is not great, however. The green segregates were destroyed within a few days after the green parent.

The difference in acceleration of aphid reproduction on the two classes is again shown in table 2. Here it is plain that in each instance the yellow plants were more resistant than the green, as is evidenced by the differences in the mean gain on comparable dates, the cumulative difference in the gain in numbers on the green as compared with the yellow, and the range of aphid number on one class as compared with that on the other. The critical ratio, as shown in the statistical treatment of the data (table 3), increased each time the counts were made.

SUMMARY AND CONCLUSIONS

The object of this study was to learn more about the observed resistance of certain varieties of canning peas to the pea aphid and the apparent relation between resistance to aphid attack and color of foliage.

Three varieties of canning peas, Yellow Admiral, Onward, and Perfection were hybridized with each other making all possible combinations. Perfection, which is susceptible, has dark-green foliage while that of the resistant Yellow Admiral and Onward is a light green which closely approximates yellow. Yellow Admiral has a tall habit of growth. The other two varieties are dwarf. The offspring were classified in the F_2 as to height of vine and color of foliage. Color variation in the progenies was continuous and only those which were obviously either yellow or green were used in the subsequent tests. Third-generation families of the different classes were grown in solid blocks and artificially infested with aphids when about 3 inches high. Four aphid counts were made from each class at 2-day intervals after the plants were in full bloom. These counts showed that classes with green foliage were susceptible and that those with yellow foliage were resistant in both classes of vine height. Fifth-generation dwarf families were tested as the third generation had been, except that each family was tested separately. All the yellow families in this test were resistant while the green families were susceptible.

The tests have shown that, among the families examined, those with yellow foliage were resistant to the pea aphid while those with green foliage were susceptible. Since this relation has held through succeeding generations, it is assumed that resistance and susceptibility are inherited with the yellow and the green foliage color respectively. In every instance the plants were artificially infested with the same number of aphids. The ensuing aphid population is, therefore, an expression of ability to resist the attack of the insect rather than an instance of tolerance or avoidance.

EARLINESS OF SEXUAL REPRODUCTION IN WHEAT AS INFLUENCED BY TEMPERATURE AND LIGHT IN RELATION TO GROWTH PHASES[1]

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INTRODUCTION

This paper deals with temperature and the photoperiod in relation to growth phases, earliness of sexual reproduction, and seasonal growth habit of the common wheat plant. In addition data are presented on the effects of two types of electric light used to lengthen the natural day in relation to the time of sexual reproduction and the number of seeds produced.

Part of the data here presented support portions of a note (*25*) [2] and of a previous paper (*27*) by the writers.

REVIEW OF LITERATURE

So far as the writers are aware, Adams (*2*) was the first to point out the importance of temperature in relation to the daily photoperiod in regulating the time of sexual reproduction in wheat. He claimed that these two factors are interchangeable. While the results of his tests suggest a compensatory relationship, they do not prove the point, since temperature was not varied concurrently with the length of the daily photoperiod. He obtained his different temperatures by conducting the tests during different periods of the year. The following year Adams (*3*) continued his study of temperature and the photoperiod, but he carried out no experiments with wheat that gave evidence on the interchangeable relationship between temperature and the photoperiod.

Enomoto (*9*) conducted experiments that showed the importance of both temperature and the photoperiod in relation to earliness in wheat and barley. The writers (*25*) presented brief data on temperature and the photoperiod and indicated their compensating relationship with respect to earliness in wheat. Maximov (*31*) indicated the same relationship with respect to wheat in a statement, but he presented no data. Hurd-Karrer (*19*) has shown the influence of temperature and the photoperiod on a spring wheat and of the photoperiod on a winter wheat. Several workers have studied temperature (*17, 21, 22*) and the photoperiod (*8, 10, 13, 30, 43*) independently in relation to the development of the wheat plant.

The majority of the environmental studies that have been conducted under controlled conditions have been managed so as to maintain as nearly constant conditions as possible throughout all the growth

[1] Received for publication June 1, 1935; issued December 1935.
[2] Reference is made by number (italic) to Literature Cited, p. 639.

phases of the plant, little or no attention being given to special requirements of the plant at different stages of development. This is the orthodox method, and while it is reasonably satisfactory for the study of certain types of plants such as the spring cereals, it is not satisfactory for many important studies on such plants as the winter cereals.

The literature shows that the temperature relations of the growth phases in winter cereals, especially the stimulating influence of low temperature during early germination on subsequent earliness in winter wheat, were recognized many years ago. However, this knowledge was not widely accepted, and it appears that these relationships have been lost sight of and rediscovered several times since the first record.

The writers (*27*) pointed out that Klippart (*23, p. 757*) knew before 1857 that low temperatures applied to germinated seed induced winter wheat when sown in the spring to mature and produce a good crop. Recently Martin [3] pointed out that this phenomenon was noted in winter wheat as early as 1837 by a grower in New York (*1*), but as irregular heading was reported by this grower it is apparent that the time of exposure to low temperatures was too short or that germination incomplete during the period of low temperatures. In 1850 Allen (*4, p. 128*) made a similar report in his book, but he thought germination unnecessary before chilling. He was not aware that germination had started in the soaked seeds held near freezing. Klippart's report indicates that he had a better understanding of the importance of germination and of the exposure time than did those before him, but he and those before him did not seem to understand that freezing temperatures are less effective (*27*) than temperatures slightly above the freezing point. Recently this and other methods of accelerating sexual reproduction have been designated in Russia by the term "iarovization", meaning vernalization, the English equivalent. Literature and experiments on this subject are dealt with in other papers [4] (*6, 26, 28, 44*).

Hellriegal (*16*), working with barley advanced beyond the early germination stage, concluded that this plant has a lower optimum temperature during the tiller-formation phase than during the stem-elongation phase. Gassner (*15*), Maximov and Pojarkova (*33*), Pojarkova (*37*), and Papadakis (*35*) concluded from experimental data that earliness in winter wheat is favored when low temperatures obtain during the seedling stage and when higher temperatures obtain during the later stages of growth.

In studies on the photoperiod Wanser (*43*) concluded that the several growth phases of the plant have different length-of-day requirements. Rasumov (*39*) studied the influence of short days followed by long days, long days followed by short days, continued short days, and continued long days on earliness in millet, spring oats, and spring barley, and he conducted field plantings with spring wheats. No tests with winter wheat were reported. With millet Rasumov found earliness of sexual reproduction favored equally by the continued short day and by 10 short days followed by 6 long days. His data on oats and barley show that the constant long days

[3] MARTIN, J. H. IAROVIZATION IN FIELD PRACTICE. U. S. Dept. Agr., Bur. Plant Indus. 13 pp. 1934. [Mimeographed.]

[4] —— Martin, J. H. See footnote 3.

favored earliness. A test carried out by Hurd-Karrer (*18*) indicated that a short day followed by a long day favored early jointing in Turkey winter wheat. The writers (*25*) presented preliminary data showing that early sexual reproduction in winter wheat is favored by an initial exposure to short days followed by long days. Maximov (*31*) drew the same conclusion but presented no data. In a later paper summarizing work on temperature and the photoperiod, Maximov (*32*) erroneously stated that the writers (*27*) found that shortening the day at the higher temperatures during the first week of growth made it possible for winter cereals to fruit the first year. The writers' studies indicate that an exposure of but 1 week is too short to induce more than an exceedingly slight acceleration of earliness in the winter wheats studied. Forster et al. (*10*) conclude that an initial short day hastens maturity in winter wheats.

In addition to the environmental studies made under controlled or semicontrolled conditions, there have been a number of studies based on date-of-seeding tests in the field, a few of which are cited (*5, 11, 14, 24, 36*).

METHODS AND TERMINOLOGY

The experiments were conducted during the winter and spring seasons in culture chambers or in greenhouses, and some tests were conducted outdoors during the summer. The plants were grown in good soil in earthen pots 8 inches in diameter. Each pot supported two plants. Care was exercised in maintaining uniform soil moisture throughout a given test by frequent applications of water.

In certain tests the seeds of Harvest Queen winter wheat were germinated and chilled at 26° to 32° F. in a dark mechanical refrigerator for several weeks before planting, as indicated.

Days longer than the natural day were obtained by means of electric lamps turned on at sunset. Except when otherwise stated, the electric source consisted of Mazda C tungsten lamps which gave an intensity ranging from 20 to 40-foot candles at the soil line. The length of the natural day was taken as from middawn to midtwilight, an increase of 1½ to 1¾ hours over a natural day length taken as from sunrise to sunset. Days longer than the natural day were computed from middawn. The 8-hour day started at 8 a. m. and ended at 4 p. m.

The initial growth phase of winter wheat, which, under cultural conditions, takes place in the autumn and winter, has commonly been referred to as the rosette phase or as the resting or dormant stage. However, it has been pointed out that this is not always a true resting stage and that a resting or a dormant condition during early growth is not essential for sexual reproduction in winter wheat (*10, 22, 42*). These conclusions are borne out by the writers' studies. In regions of very cold winters the plants are dormant during the winter period, but at Rosslyn, Va. (near Washington, D. C.), growth continues slowly throughout the winter except in an occasional year when there may be a short dormant period. In this paper this stage is referred to as the "leaf-and-internode-formation phase", and the term is applied to winter and spring varieties. Hellriegal (*16*) used a similar terminology for barley. The so-called jointing stage is designated the "stem-elongation phase."

All data on earliness are based on the date when the tip of the head reached a point as high as the ligule of the flag leaf. Fertilization occurred 2 to 4 days after heading.

Further details on methods are taken up throughout the paper under the several experiments.

EXPERIMENTAL RESULTS

TEMPERATURE AND THE PHOTOPERIOD IN RELATION TO THE GROWTH PHASES AND SEXUAL REPRODUCTION

During the winter of 1928–29 a test was conducted with Harvest Queen, a typical winter wheat. Plantings were made in a greenhouse with germinated seeds that had been held in a refrigerator for 67 days at 30° to 35° F. Unchilled germinated seeds were planted as controls. Some of the plants were exposed to the natural day, which ranged from 11 hours at the beginning of the test to 15 hours at the end, and others to a long day, ranging from 16 to 17 hours. Mazda C tungsten lamps were used for supplementing the natural day. The temperature of the greenhouse was usually about 55° to 65° F., though on bright days the temperatures went higher.[5]

Table 1 and figure 1 show that of the plants from the chilled seedlings, those under the long day headed first; of the plants from the unchilled seedling, those under the short day headed first; under both the long and the short day, the plants from the chilled seedlings headed earlier than the plants from the unchilled seedlings. This test shows the stimulating influence of low temperatures during early germination, in the absence of light, on subsequent earliness of sexual reproduction in long days with high temperatures.

TABLE 1.—*Period from planting to first heading in Harvest Queen wheat as influenced by chilling in darkness during germination and by the length of the photoperiod after chilling in comparison with plants from seed not chilled before planting*

[Seed was planted Dec. 18, 1928]

Length of daily photoperiod after planting	Germination temperature 30° to 35° F. for 67 days in darkness, followed by 55° to 65°		Germination temperature 70° F. for 4 days in darkness, followed by 55° to 65°	
	Period from planting to heading	Tillers per plant when internodes started to elongate	Period from planting to heading	Tillers per plant when internodes started to elongate
Hours	*Days*	*Number*	*Days*	*Number*
11 to 15	118	5.1	125	12.2
16 to 17	66	2.9	128	12.9

Since the length of day in the short-day series was 4 hours shorter during early growth than during later development, it seemed probable that this initial short day was a factor in the earlier heading noted in the plants from the unchilled seedlings. The following winter this relationship was studied further with Harvest Queen winter wheat and Purplestraw, a facultative wheat.[6] As stated in a note (*25*), a 7-

[5] This statement was inadvertently omitted in a previous publication (*27*).

[6] The writers have discussed the basis of distinction between spring, facultative, and winter varieties of wheat in another publication (*27*).

to 8-hour day during the early development of the plant favored earliness slightly in Harvest Queen, but this short day retarded heading slightly in Purplestraw.

During the same winter another test was carried out with Harvest Queen wheat and with Marquis, a spring wheat. The seeds were germinated but not chilled before planting. Some of the plants of

FIGURE 1.—Harvest Queen winter-wheat plants: *A* and *C*, From germinated seeds chilled at 30° to 35° F. in the dark for 67 days; *B* and *D*, from seeds that were not chilled; *A* and *B*, grown with a daily photoperiod of 11 to 15 hours; *C* and *D*, grown with a daily photoperiod of 16 to 17 hours.

each variety were grown at low temperatures in plant-culture chambers during the first 54 days, and later they were given high growing temperatures in a greenhouse. Other plants of each variety were grown at high temperatures in a greenhouse throughout the test. Short, medium, and long days were maintained during the first 54 days at the low and high temperatures (table 2).

Table 2 and figures 2 and 3 show that the longest days at the high temperatures from the beginning of growth induced the earliest heading in Marquis spring wheat (46 days) and the latest heading in Harvest Queen winter wheat (155 days). The shortest days at the low temperatures during the first 54 days induced the earliest heading in

FIGURE 2.—Marquis spring-wheat plants (table 2): *A*, *B*, and *C*, Grown in a greenhouse at temperatures averaging near 70° to 75° F. and above; *D*, *E*, and *F*, grown in a culture chamber near 51° for 36 days, followed by temperatures near 59° for 18 days, followed by 70° to 75° and higher; *A* and *D*, with daily photoperiod 8 hours long for first 54 days; *B* and *E*, with daily photoperiods 12 to 14½ hours long for first 54 days; *C* and *F*, with daily photoperiods 16¼ to 17¾ hours long for first 54 days; subsequent daily photoperiods, 17¾ to 19 hours.

Harvest Queen (88 days) and the latest heading in Marquis (88 days). Fertilization started 2 to 4 days after heading in both varieties. These results, like those of Garner and Allard (*13*), indicate that Marquis spring wheat is a long-day plant with respect to earliness of sexual reproduction.

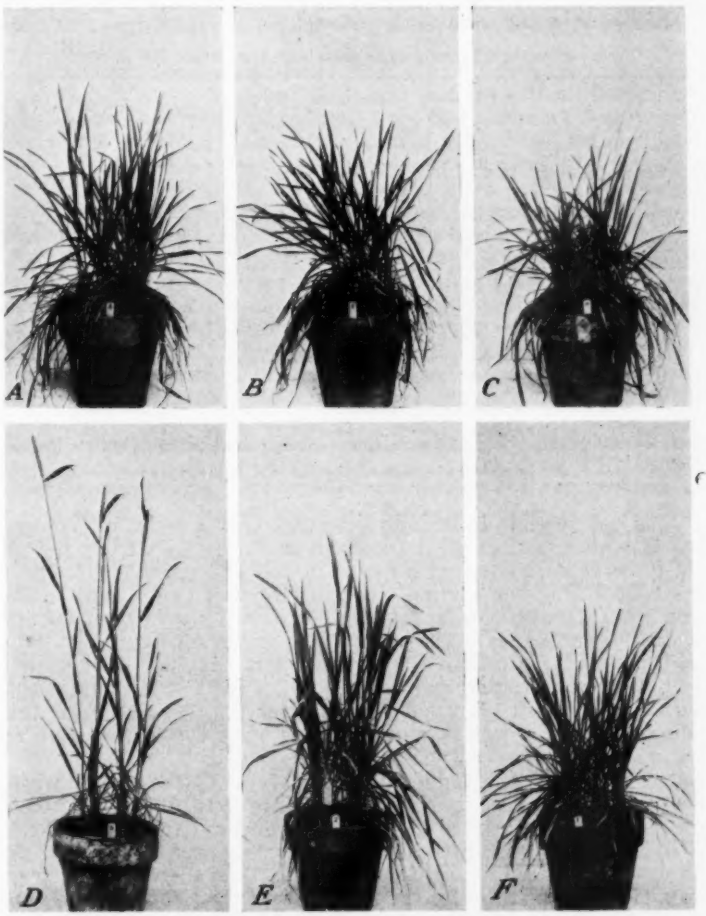

FIGURE 3.—Harvest Queen winter-wheat plants (table 2): *A*, *B*, and *C*, Grown in a greenhouse at temperatures averaging near 70° to 75° F. and above; *D*, *E*, and *F*, grown in a culture chamber near 51° for 36 days, followed by temperatures near 59° for 18 days, followed by 70° to 75° and higher; *A* and *D*, with daily photoperiod 8 hours long for first 54 days; *B* and *E*, with daily photoperiods 12 to 14½ hours long for first 54 days; *C* and *F*, with daily photoperiods 16¼ to 17¾ hours long for first 54 days; subsequent daily photoperiods, 17¾ to 19 hours.

It is evident from the data and the illustrations that the short days and the low temperatures during early growth (the leaf-and-internode-formation phase), followed by the longer days and higher temperatures, favored the earliest sexual reproduction in the winter variety,

thus indicating that the early growth phase has different temperature and photoperiodic optima from those of the subsequent growth phases.

TABLE 2.—*Heading time in Marquis spring wheat and Harvest Queen winter wheat as influenced by low and high growing temperatures and by short, medium, and long daily photoperiods during the first 54 days, after which all plants received high temperatures and long days as indicated*

[Seeds were soaked and germinated for 2 days before being planted Feb. 12, 1930]

Variety and photoperiod [1]	Temperature, 51° F. for 36 days; then 59° for 18 days; subsequently 70° to 75° and above			Temperature, 70° to 75° F. for 54 days; subsequently 70° to 75° and above		
	Period from soaking of seed to heading	Tillers per plant	Heads per plant	Period from soaking of seed to heading	Tillers per plant	Heads per plant
	Days	*Number*	*Number*	*Days*	*Number*	*Number*
Marquis:						
8 hours	88	14.5	8.0	78	8.5	4.0
12 to 14½ hours	83	8.5	6.2	76	12.5	4.0
16¾ to 17¾ hours	65	8.2	7.2	46	4.5	4.0
Harvest Queen:						
8 hours	88	17.7	4.2	[2] 107	19.2	4.7
12 to 14½ hours	95	22.5	5.2	[2] 134	21.2	2.0
16¾ to 17¾ hours	[3] 114	22.5	4.7	[2] 155	22.5	3.0

[1] After 54 days all daily photoperiods were maintained at 17¾ to 19 hours.
[2] Many of the tillers on these plants did not head, but this condition was less marked than in the plants designated in footnote 3.
[3] All plants were very vegetative and heading was slow, requiring 50 to 60 days for completion in the case of some plants. On Aug. 15 some plants still were producing tillers that were jointing and heading.

It was not possible to include simultaneously a series of temperatures and photoperiods held constant or nearly so during the life cycle to serve as additional controls in this experiment. However, other tests have been carried out with Harvest Queen and similar winter wheats grown constantly near 60° F. and also at temperatures between 60° and 75° in constant long days and in constant short days. In all instances, more than 100 days to more than 200 days elapsed from planting to heading and fertilization. Tests of Hutcheson and Quantz (*21*), Klages (*22*), and Hurd-Karrer (*17*, *19*) also show that heading in winter wheat is relatively slow when the temperature requirements of the growth phases are disregarded.

An experiment was conducted with Harvest Queen winter wheat during midsummer with daily photoperiods of 8, 15, 18, and 24 hours. Each photoperiod remained unchanged throughout the test. The 8- and 15-hour days comprised sunlight only, whereas the 18- and 24-hour days comprised sunlight for 15 hours and electric light from Mazda C tungsten lamps for the remainder of the photoperiod. The seeds were soaked in water and germinated before planting, but they received no low temperatures. The plants given 18 and 24 hours of light daily headed 129 and 110 days, respectively, from the time the seeds were placed in water. No heads were produced by plants given 8 and 15 hours of light daily up to the time the test was terminated on November 11—178 days from the beginning of the test.

These results by themselves would lead to the conclusion that winter wheat is a typical long-day plant with respect to early sexual reproduction, as was concluded by Forster et al. (*10*) from similar

tests, and by Hurd-Karrer and Dickson (*20*) from tests (*19*) conducted in a cool greenhouse during the winter. However, in table 2 it will be noted that Harvest Queen headed in 88 days when the plants were grown with a daily photoperiod of 8 hours at low temperatures followed by long photoperiods at high temperatures. Thus heading was 22 days earlier with this treatment than it was when continuous light and high summer temperatures obtained from germination to heading.

Under the conditions of Hurd-Karrer's (*19*) test, flowering started in Turkey winter wheat 188, 165, and 132 days from planting when grown with daily photoperiods of 8, 9½ to 15, and 17 hours, respectively, and with the temperature held at 12°±1° C. throughout the test. With the same strain of Turkey the writers (*27*) found that flowering started 113 days from planting when moderately low temperatures and short days preceded higher temperatures and the long day. In later tests, this strain of Turkey started to flower 105 days after planting, when the temperatures and exposure periods were nearer their optima. Other varieties of winter wheat have been tested and found to head and flower sooner when suitable low temperatures and short days were followed by suitable high temperatures and long days.

The results of more than 500 dissections of many varieties of winter and spring wheats indicate that the first signs of differentiation of the floral organs become evident soon after the last leaf primordium is differentiated, and that the internodes of the stem start their major elongation a little before or simultaneously with the differentiation of the floral organs.

The number of leaves and stem internodes formed by a shoot in winter wheat as well as in spring wheat (*27*) is influenced by temperature and the photoperiod. Harvest Queen wheat has produced as few as 7 internodes[7] per shoot when fully germinated seeds were first chilled for 67 days in darkness and subsequently grown with continuous light at summer temperatures. Heading started 33 days after the completion of the chilling process. As many as 22 internodes per shoot have been produced by this variety when the seed was not chilled and when the plants were grown in uninterrupted light at warm temperatures, and under these conditions heading started 110 days after planting. Similar results were obtained when a continuous 8-hour day and warm temperatures were maintained from the beginning of germination. In field culture Harvest Queen has been observed to form 12 or 13 leaves and internodes on the primary stalk. When small numbers of internodes are formed most of them elongate, but when large numbers are formed many fail to elongate appreciably. The early cessation of the formation of leaf primordia in Harvest Queen wheat does not take place during the exposure to the low growing temperatures and short days, but during the subsequent exposure to the high temperatures and long days.

To classify accurately varieties of wheat with respect to their earliest sexual reproduction in relation to temperature and photoperiod it is necessary to determine the optimum temperatures and

[7] Only the internodes above the subcrown internode were included, and these were determined by counting the leaves.

optimum photoperiods for the formation of a reduced number of stem-internode primordia, the early differentiation of the floral primordia, the rapid elongation of the internodes, and the rapid development of the floral organs and maturity. It seems reasonable to believe that ultimately a graphic representation of the optimum temperature and photoperiod characteristic of a given variety of winter wheat can be expressed in the form of more or less smooth gradient curves. However, until more perfect methods are available, approximations must suffice.

In view of the evidence based on present methods for approximating these optima, it seems justifiable to conclude that Harvest Queen, Turkey, and similar winter wheats are not typical long-day plants with respect to their earliest sexual reproduction, but are what may be termed short-day→long-day plants, and they may be considered as low-temperature→high-temperature plants. This method of expression indicates that the temperature and the photoperiod must increase with the development of the plant in order to induce early sexual reproduction. A similar situation seems to apply to the facultative varieties and to certain late varieties commonly placed in the spring group (*27*), but in these the initial optimum temperatures are higher or the periods of exposure to low temperatures are shorter than is the case with the strictly winter varieties (*27*).

None of the above tests was planned for yield determinations. However, tiller counts are recorded in tables 1 and 2 and head counts in table 2. Conditions favoring earliness favored a reduction in the number of tillers. Observations indicated that Marquis produced larger and more completely filled heads when the initial temperatures were low. In Harvest Queen the plants receiving the initial low temperatures with the initial 8-hour day produced an average of 75 seeds per plant, and these plants were similar to the average Harvest Queen plant grown in the field. Although no seed counts were made on the remaining Harvest Queen plants, it appeared from observations that the greatest number of seeds was produced by the plants receiving the initial low temperatures with the medium length of day, and these plants were considered to exceed the normal. The continuous high temperature and the initial long day at the initial low temperatures favored a large number of tillers in Harvest Queen, but the few heads produced were relatively small and poorly filled and the plants were decidedly abnormal in all respects in comparison with the field type.

MISCELLANEOUS TESTS RELATING TO CHILLING OF SLIGHTLY GERMINATED SEED

Darkness v. Light During Chilling Period

An experiment was carried out to determine if darkness during the chilling treatment has any stimulating influence on subsequent earliness. Germinated seeds of Turkey winter wheat were chilled for 61 days at 38° F. Some of the seeds were in total darkness and others received daylight. After chilling, growth continued outdoors at summer temperatures with a daily photoperiod of 17 to 18 hours. The plants from seed chilled in daylight headed 47 days after planting, and those from seed chilled in darkness headed in 49 days. From these results it seems evident that the low temperature is the stimulating agent for subsequent early heading.

Importance of Moisture and Germination During Chilling Period

An experiment was carried out with dry and with moist ungerminated seeds, with seeds that were germinated and then dried and with seeds that were germinated and kept moist during the process of chilling. All seeds were chilled for 65 days near 34° F. After being chilled the seeds were planted with an unchilled control at temperatures near 70° to 75° and photoperiods that ranged from 16 to 18 hours.

Plants from dry seeds, whether germinated or not, and from unchilled seeds had produced no heads when the test was discontinued 150 days after planting. Plants from seeds that were germinated and kept moist during the chilling process headed in 45 days. The plumules and radicles of seeds kept moist but not germinated before the beginning of the chilling process were showing slight activity at the end of the chilling treatment. Plants from these seeds started to head in 135 days, but heading was irregular.

Stage of Development of the Seed

A test was carried out with Harvest Queen seed in the soft-dough stage and in the hard-dough stage and with completely ripened seed 1 year old, to determine whether low temperatures during germination stimulate subsequent early heading in plants from seeds in the soft-dough and in the hard-dough stage. All seeds were first germinated at 60° F. and then chilled near freezing for 72 days. After chilling, growth continued at 68° to 90° with a daily photoperiod of 16 to 17½ hours. Plants from seed in the soft-dough stage headed in 43 days, those from seed in the hard-dough stage headed in 45 days, and those from the old seed headed 43 days from the date of planting, thus showing that seed in the soft-dough and hard-dough stages reacts efficiently to the chilling treatment. This permits a more rapid increase of winter-wheat populations in genetic work.

Earliness and Tiller Reduction

When germinated seeds have been chilled sufficiently, several tillers ultimately joint and produce heads relatively quickly at suitable temperatures and day lengths, thus indicating that the function altered by the low temperature is not confined entirely to the plumule (primary shoot).

It was thought that the effects of chilling on early stem elongation and heading at high temperatures might be due directly to the reduction in the number of tillers, which is correlated with the early heading of plants from chilled seedlings. If this were true, another method of reducing tillers might accelerate stem elongation and heading. To test this theory, several Harvest Queen plants from unchilled seedlings were grown outdoors during the summer of 1931. Some of these plants were allowed to grow in the usual manner, whereas others were not allowed to produce more than one shoot each, all extra shoots being cut from the plants as soon as observed. The single shoots developed very long, wide dark-green leaves and very large stems, in comparison with the unpruned plants. However, the fact that pruning did not stimulate early heading indicates that the reduced tillering in plants from chilled seedlings is not the direct cause of early heading.

Localization of the Region of the Functional Changes Induced by Low Temperatures

In an attempt to localize the region in which the functional changes occur at low temperatures, seeds of Kanred winter wheat were germinated until the roots were 2 mm long; the roots and plumules were then cut off before the initiation of the chilling process, care being taken to remove the tips and as much of these organs as possible without total destruction of the embryo. These and unmutilated seedlings were chilled near freezing in the dark for 65 days. At the completion of the process the seedlings were given continuous light at 70° to 80° F. All the mutilated seedlings headed at the same time as the unmutilated controls. It is evident, therefore, that the active region in question is not confined to the apical regions of the seminal roots, the coleoptile, or the first leaf. It is also apparent that this active region is not confined to the endosperm, because under field conditions at the Arlington Farm the endosperm frequently is completely absorbed in early autumn before the onset of cold weather, and under these conditions the winter wheat plants mature normally the following summer.

Influence of Intensity of Light from Tungsten Lamp

Since the Mazda tungsten lamp is commonly used for lengthening the natural day, a test was conducted to determine the influence of intensity of this artificial source of light during the winter and spring months. Marquis, Siberian No. 1, and Romanov spring wheats were used. Each variety was grown in earthen pots 8 inches in diameter, each pot containing two plants. The pots were placed in 3 parallel rows 12 feet long on a bench in a small glasshouse, pots containing plants of the same variety being placed in the same row.

Two 150-watt Mazda C tungsten lamps in a large reflector were placed at one end of the bench but not directly over the first pots. The plants farthest from the lamps, therefore, received less light at night than those nearest the lamps. The lamps were turned on at sundown and off at sunrise each day during the test, after which daylight only was provided, giving the plants a continuous exposure to light.

The intensity of the light from the lamps was determined during the night at three distances above each pot in the center row, as indicated in table 3. The test plate of a Macbeth illuminometer was held at right angles to the shortest distance between the light source and the center of the plate. Owing to the shape of the lamp reflector and to shadows from intervening plants, the intensities reduced more rapidly than is provided for in the inverse-square-distance law.

Care was exercised in maintaining a uniform temperature near 70° F. throughout the experiment by means of oscillating fans. As spring advanced there was a rise in temperature outdoors, but the uniformity of the temperature was maintained throughout the greenhouse.

TABLE 3.—*Influence of intensity of Mazda C tungsten lamps on 3 varieties of wheat when grown near 70° F. during the short days of winter, the tungsten source being used from sunset to sunrise, making continuous illumination*

[Test started Dec. 8, 1931]

Pot no.	Intensity of lamps at indicated heights above soil line over each pot in center row			Heading time			Culms per plant			Average final height of plants		
	4 inches[1]	18 inches[2]	28 inches[3]	Siberian No. 1	Marquis	Romanov	Siberian No. 1	Marquis	Romanov	Siberian No. 1	Marquis	Romanov
	Foot-candles	*Foot-candles*	*Foot-candles*	*Days*	*Days*	*Days*	*Number*	*Number*	*Number*	*Inches*	*Inches*	*Inches*
1	76.3	100.0	272.5	39	48	50	3	8	6	20	26	31
2	56.7	63.0	93.7	42	51	50	4	14	12	22	28	31
3	38.1	25.0	38.1	43	51	51	5	10	9	21	26	31
4	25.1	12.0	22.9	42	51	52	5	11	7	21	27	31
5	20.0	9.0	15.3	43	53	53	4	10	9	23	23	34
6	8.7	5.0	7.0	45	52	56	5	9	12	17	28	29
7	6.8	4.3	5.4	45	53	53	4	10	12	19	28	37
8	5.8	2.0	4.0	50	63	63	6	18	11	22	28	37
9	4.5	1.2	3.4	50	64	64	8	11	12	27	25	39
10	3.6	.7	2.8	52	69	69	9	8	8	21	31	42
11	3.1	.65	2.4	59	75	76	16	10	7	27	32	43
12	2.5	.5	2.0	69	91	91	14	11	10	34	33	45

[1] When this reading was made the plants did not cast a shadow on adjacent plants.
[2] When this reading was made the plants ranged from 20 to 28 inches tall, and the test plate was shaded by adjacent plants except in the case of the first plant in the row.
[3] When this reading was made the test plate was not shaded by adjacent plants.

The data in table 3 show that earliness was favored by the more intense light. In general, the differences in response of the plants became most evident when the light intensity at 28 inches above the soil line during the night period reached two foot-candles and less. Siberian No. 1 produced the least number of tillers near the lamps and the greatest number when at the greatest distance from the lamps. Tillering in the other varieties did not vary appreciably as the light intensity was varied. The final height of the plants was greatest in plants farthest from the lamps. Romanov, with slight exceptions, showed the most consistent gradual increase in height as the light intensity was reduced.

Leaf and straw sizes were greatest when the supplementing light intensity was reduced. The leaves averaged five-sixteenths of an inch wide by 11 inches long near the lamps and one-half of an inch wide by 18 inches long farthest from the lamps. The largest nodes on plants near the lamps averaged 0.107 inch in diameter, whereas those of plants growing farthest from the lamps averaged 0.175 inch in diameter.

Romanov and Marquis heads did not fill well, whereas Siberian No. 1 filled well in all intensities of light. Many heads on Marquis plants contained but two or three kernels.

The most rapid stem elongation occurred in the plants that were first to head (fig. 4), but the plants that headed last continued their growth for a longer period and at maturity were much taller than the ones that headed first (table 3).

FIGURE 4.—Romanov spring wheat grown near 70° F. with uninterrupted light during the winter and early spring months. Mazda C tungsten lamps were turned on at sunset and off at sunrise; during the day all plants received equal intensities of sunlight; during the night different plants received different intensities of light. Pots *A*, *B*, and *C* correspond to pots 1, 6, and 12 in table 3.

Although the intensity of the supplementing light influences earliness and other responses of the plant, the data presented indicate that changes did not become marked until the light intensity had changed to a relatively great extent. This is borne out also in Garner and Allard's (*12*) and Shirley's (*41*) studies with several species, and in Hurd-Karrer and Dickson's (*20*) studies with wheat.

The Mazda C tungsten lamp was compared with summer sunlight in a test with Marquis spring wheat. This variety was grown with a daily photoperiod of 15 hours outdoors during midsummer. One series received direct sunlight alone and another received 8 hours of direct sunlight supplemented by 7 hours of light from a Mazda C tungsten lamp delivering 8 to 10 foot-candles at the soil line.

Plants receiving full sunlight produced 14.2 tillers per plant and headed in 50 days, whereas those receiving both sunlight and electric light produced only 7.3 tillers per plant and headed in 46 days.

It seems likely that a reduction in the light intensity during part of the day, as well as the spectrum characteristic of the Mazda C tungsten lamp, caused the rapid completion of the vegetative period and sexual reproduction in this test. This lamp is especially strong in the yellow, orange, red, and infrared (*7*). Yellow, orange, and red have been reported to favor rapid development in certain plants (*34*, *38*).

The retarding influence of bright sunlight on plant growth has been observed by others (*40*, *41*). In the writers' tests, reducing summer sunlight to 41.3 percent reduced the heading time in Marquis to 50 days as compared with 55 days in unobstructed sunlight. Reducing the light to 27.9 percent increased the heading time to 58 days, and no heading took place when the intensity was reduced to 11.5 percent. The stems elongated most slowly in unobstructed sunlight, but the heads were more numerous and better filled than in the shaded series. Temperatures were equalized by means of fans.

COMPARISONS BETWEEN TUNGSTEN LAMP AND COOPER-HEWITT WORK LAMP

Tests were carried out for the purpose of comparing clear Mazda C tungsten lamps with a Cooper-Hewitt mercury-arc lamp having a lead-glass tube 50 inches long, as sources of light for lengthening the autumn and winter days.

The Mazda C lamp, as stated above, is especially strong in the infrared, red, and yellow, whereas the Cooper-Hewitt lamp emits yellow, green, blue, and violet, the red and orange being absent. The foot-candle intensities of the lamps were determined by means of the Macbeth illuminometer. Special screens were used in the illuminometer to procure this information for the Cooper-Hewitt lamp. Of the two factors (0.2 and 0.5) recommended for computing the foot-candle intensity of the Cooper-Hewitt lamp, the former was used. Two tungsten bulbs were used in white reflectors in order to obtain a uniformity of light distribution approaching that of the Cooper-Hewitt lamp. The plants received 8 hours sunlight and 8 hours artificial light daily. Temperatures ranged from 68° to 77° F.

Marquis spring wheat and Harvest Queen winter wheat served as test plants. Both chilled and unchilled seeds of Harvest Queen were used.

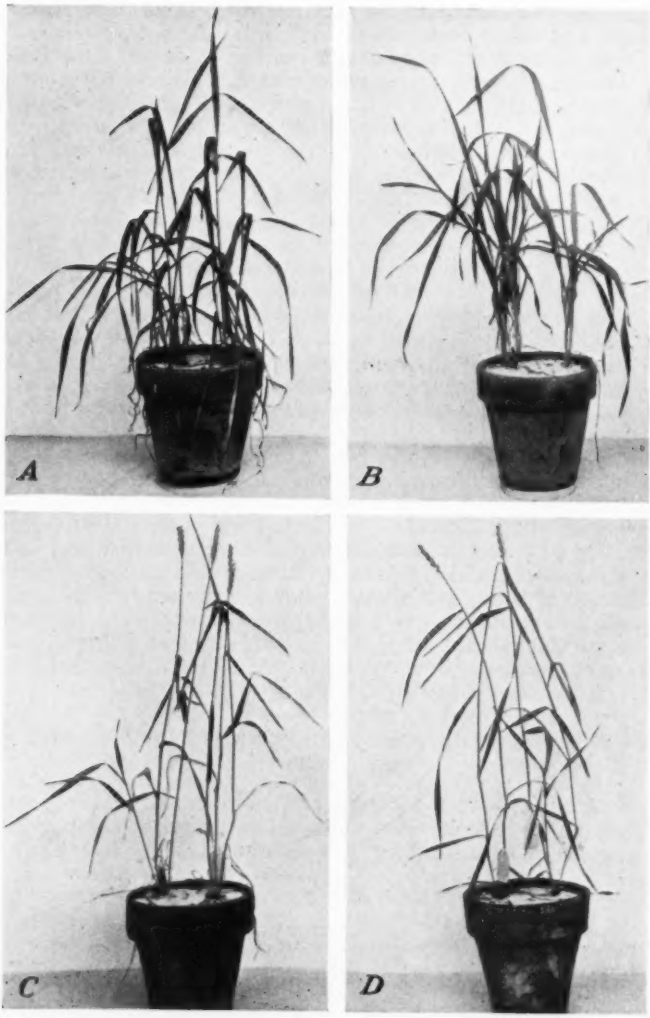

FIGURE 5.—*A* and *C*, Marquis spring wheat; *B* and *D*, Harvest Queen winter wheat from chilled seeds. *A* and *B*, grown with 8 hours of daylight and 8 hours of light from the Cooper-Hewitt mercury-arc lead-glass-tube lamp daily; *C* and *D*, grown with 8 hours of daylight and 8 hours of light from the Mazda C tungsten lamp daily.

It will be noted in table 4 and figure 5 that the tungsten source had a greater accelerating influence on the time of heading than did the Cooper-Hewitt lamp, but more tillers were produced under the Cooper-Hewitt lamp. Seed yields were not obtained, as mice destroyed some of the heads, but observations indicated that more heads and more seeds per head were produced in the plants grown under the Cooper-Hewitt lamp.

Mason (*29*), working with the date palm, observed that the Mazda lamp favored the pushing of the leaves from the growth center, whereas the Cooper-Hewitt lamp had an inhibiting influence.

TABLE 4.—*Period from planting to heading as influenced by the Mazda C tungsten lamp and the Cooper-Hewitt mercury-arc lamp with lead-glass tube, when used to supplement sunlight* [1]

[Planted Sept. 19, 1930]

Variety and treatment of germinated seed	Mazda C tungsten lamp (44.7 foot-candles)	Cooper-Hewitt lamp (69.7 foot-candles)
	Days	*Days*
Marquis:		
Seed not chilled	50	81
Harvest Queen:		
Seed chilled for 65 days	50	85
Seed not chilled	(2)	(2)

[1] Lamps operated 8 hours before sunrise, followed by sunlight alone for 8 hours. Temperatures ranged from 68° to 77° F.

[2] There was no sign of stem elongation at the end of 90 days.

Seed yields were obtained in another test, however. Marquis spring wheat and five varieties of winter wheat from chilled seedlings were grown in a greenhouse near 70° to 75° F. during late autumn and winter. For the first 36 days the natural photoperiod of about 11 hours was maintained. After this period uninterrupted light was provided. In one-half of the series, daylight was supplemented with artificial light from the Cooper-Hewitt work lamp; in the other half, Mazda C tungsten lamps were the supplementary source. The lamps were turned on at sunset and off at sunrise. The light intensities in both tests were maintained as nearly as possible at 60 foot-candles at a point midway between the soil and the top of the plant.

In table 5 it will be observed that when the Cooper-Hewitt lamp was used the seed yield was increased in all varieties except White Winter. The White Winter plants were less uniform in their growth than the other varieties, and this may account for their response. There were not many plants in the winter varieties, but there was a reasonably large number in Marquis.

TABLE 5.—*Seed production as influenced by the Mazda C tungsten lamp and by the Cooper-Hewitt mercury-vapor lamp with lead-glass tube* [1]

[Seeds were planted Oct. 13, 1931]

Variety	Plants under—		Heads produced under—		Seeds per head produced under—	
	Mazda C tungsten lamp	Cooper-Hewitt lamp	Mazda C tungsten lamp	Cooper-Hewitt lamp	Mazda C tungsten lamp	Cooper-Hewitt lamp
	Number	*Number*	*Number*	*Number*	*Number*	*Number*
Harvest Queen	2	2	10	7	10.9	18.8
Crimean	2	2	10	8	9.6	16.5
Minhardi	2	2	8	7	15.7	19.4
White Winter	2	2	11	10	13.7	10.9
Soi	2	2	7	10	5.29	13.5
Marquis	20	20	28	29	1.35	2.93

[1] During the first 36 days the natural photoperiod obtained, after which uninterrupted illumination was provided, the augmenting sources operating from sunset to sunrise.

The following winter another test was carried out with F_1 plants from winter-wheat crosses. The germinated chilled seeds were planted in the greenhouse. The temperatures were about 70° to 75° F. and the daily photoperiod was about 16 hours. Mazda C tungsten lamps were used after sunset. This photoperiod was maintained until the flag leaves appeared, when the series was divided into two groups of 16 plants each. Each group contained exactly the same progenies, and the plants were selected for uniformity of development. One group was placed under tungsten lamps and the other under the Cooper-Hewitt lamp combined with tungsten lamps. The light intensities in this test were maintained as nearly as possible at 120 foot-candles at a point midway between the soil and the top of the plant. The plants received full daylight. The lamps were turned on at sunset and off at sunrise.

Seed counts were made on the primary head of each plant. The 16 primary heads under the Cooper-Hewitt and Mazda C lamp combination produced 314 seeds, and the 16 primary heads under the Mazda C lamps produced only 216 seeds. The number of seeds per head ranged from 16 to 22 under the combined lamps and from 10 to 17 under the Mazda C lamps.

Very poor seed sets have been obtained when wheat plants begin to head in December and early January. Even when the day was lengthened artificially, the heads were frequently poorly developed and fertilization usually was not normal. Tests carried out in reduced sunlight in midsummer give similar results, thus indicating that the low intensity of daylight may be the chief cause of the poor heads in midwinter. All evidence seems to indicate that the success of a long day in which artificial light is used depends to a large extent on the amount of sunlight available.

CONCLUSIONS AND SUMMARY

Sexual reproduction in the spring wheats and in the winter wheats is not dependent on a critical temperature or a critical photoperiod, as this process occurs over very wide ranges of these factors. However, the time when sexual reproduction occurs is greatly influenced by the temperature and the photoperiod.

The proper classification of wheat varieties with respect to their temperature and photoperiodic characteristics for the earliest sexual reproduction is dependent on methods that make it possible to determine the optimum conditions of temperature and the photoperiod for the rapid completion of each growth phase, from the beginning of growth through sexual reproduction.

Spring wheats such as Marquis and earlier varieties complete their life cycle quickly when given a long day and temperatures at 70° F. or above throughout the life cycle, and therefore are considered to be typical long-day high-temperature plants. On the other hand, Harvest Queen, Turkey, and other varieties of winter wheat complete their life cycle most rapidly when given a short day and low temperatures during the early stages of growth and a long day and high temperatures during the later stages of development.

In the light of these relationships the writers conclude that winter wheats are not typical long-day plants but are what may be termed short-day→long-day plants and low-temperature→high-temperature

plants. This method of expression indicates that the temperature and the length of the photoperiod must increase with the development of the plant in order to induce early sexual reproduction.

Temperatures and photoperiods favoring earliness in the winter and spring wheats favor the formation of a reduced number of internodes and leaves by each tiller. The formation of the stem internodes and leaves stops and the major elongation of the stem begins at about the time that floral differentiation becomes evident.

When the Mazda C tungsten lamp was used to lengthen the day, Marquis headed sooner than when the same photoperiod consisted of sunlight alone. Heading was earlier under the Mazda lamp than under the Cooper-Hewitt lamp when these light sources were used to lengthen the winter day.

In 5 of the 6 varieties tested the number of seeds was greater when the winter day was lengthened by means of the Cooper-Hewitt lamp alone, or when in combination with the Mazda lamp, than when the Mazda lamps were used alone for supplementing daylight. It appears that a combination of these lamps may be better for certain studies than the Mazda lamp alone for lengthening the natural day. The Mazda lamp stimulated earliness at the sacrifice of vegetation and seed yield, and the Cooper-Hewitt lamp apparently offset this by increasing vegetation and the yield of seed.

Earliness and other characteristics of the wheat plant are influenced by the intensity of the light, and while this influence is less than that of the daily photoperiod so far as concerns earliness of sexual reproduction, it is sufficient to warrant attention in experimental work.

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THE ANATOMY AND HISTOLOGY OF THE TRANSITION REGION OF TRAGOPOGON PORRIFOLIUS[1]

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INTRODUCTION

Salsify (*Tragopogon porrifolius* L.) is a biennial plant belonging to one of the most specialized groups of the Compositae. During the first year of growth it produces a fleshy primary root, a fleshy hypocotyl, and a very short stem. In its second year a flower stalk is developed from the stem which extends to a height of 2 to 3 feet and bears disks of purple flowers. The seedling has linear, lanceolate, sessile cotyledons, which carry on photosynthesis. The fruit (commonly called the seed) is a linear achene about 1 cm in length.

REVIEW OF LITERATURE

A search of the literature failed to reveal any reports dealing with the anatomy of the transition region of salsify. Van Tieghem (*6*)[2] in 1870 described very briefly the transition in a few plants of the Compositae, and Gérard (*3*) in 1881 reported the results of studies on the seedling anatomy of two members of the Compositae. One of these, *Carthamus tinctorius*, is very similar in its type of transition to *Tragopogon porrifolius* in most details mentioned relative to the root and lower hypocotyl. Vuillemin (*8*) reported studies with the stems of some of the composites and included very brief notes on the transition phenomena. Dangeard (*2*) made very general observations on types of transition and based the results of his final classification on the number of primary xylem groups in the root. Van Tieghem (*7, p. 782*) defined three general types of transition from root to stem. Compton (*1*) described several types of syncotyly in the Compositae. His study was made from an evolutionary viewpoint. Hill and De Fraine (*4*) investigated seedling structure from two general aspects—phylogeny and physiology. They state (*4*, p. 262):

> The existence of an intermediate type between diarchy and tetrarchy has been demonstrated in certain plants, e. g. *Liriodendron tulipifera, Clematis Hendersonii*, and some of the Composites; in these, lateral bundles of the cotyledons enter the hypocotyl and attempt to form the intercotyledonary poles of a tetrarch root.

Lee (*5*) reported very briefly his work on the seedling anatomy of a large number of plants belonging to the Compositae. Of these only *Heliopsis laevis* (seedling A), *Centaurea macrocephala*, and *Tragopogon dubius* seem at all similar to salsify. However, in Lee's article, as well as in most of the others cited, very little detail is given, and one cannot be certain as to the amount of similarity in structure. The present paper presents the results of a study of the anatomical and histological changes that take place in the transition region of salsify.

[1] Received for publication May 6, 1935; issued December 1935.

[2] Reference is made by number (italic) to Literature Cited, p. 654.

MATERIAL AND METHODS

The Mammoth Sandwich Island variety of salsify was used for this study. The plants were grown in the greenhouses of the Ohio State University during the summer and fall of 1934. Seedlings of various ages were used, but those 6 to 8 days old proved to be the best material for a detailed study of the root-stem transition. At this stage all the primary tissues were differentiated but no secondary thickening was present.

The specimens were fixed in formol acetic alcohol, dehydrated according to the usual method, run through chloroform, and embedded in paraffin. Serial transverse and longitudinal sections were cut 10μ to 15μ in thickness, mounted serially, and stained in most cases with safranine and fast green.

GROSS MORPHOLOGY OF YOUNG SEEDLING

The first indication of germination is the bursting of the seed coat and the hard outer covering (composed of carpel wall and receptacle) at the base of the achene, by the elongation of the hypocotyl and growth of the radicle. The elongation of the inverted V-shaped hypocotyl brings the cotyledons, which are still enclosed by the fruit coat, out of the ground. In about a day the hypocotyl straightens, and the plantlet assumes a vertical position. The walls of the achene and seed coat usually remain, partly enclosing the growing cotyledons for a day or two, then are shed completely. In the greenhouse at a temperature of 60° to 70° F. this entire process requires from 5 to 6 days. The cotyledons are parallel-veined and sessile. At their base they are undiverged, thus forming a tube surrounding the epicotyl, but are separate at almost a centimeter above the cotyledonary node. The epicotyl grows very slowly at first, and several days elapse before elongation takes place.

ANATOMY OF THE YOUNG PRIMARY ROOT

The primary xylem of the salsify root is diarch; no exceptions to this were found. As is usual in roots, the xylem and phloem are radially arranged in the protostele (fig. 1). Within about 7 days after the seed is planted the primary body is differentiated. There are 6 to 10 cells in each protoxylem group abaxially arranged with respect to the 10 to 15 central metaxylem elements. These vessels are spirally thickened both in root and hypocotyl. Several layers of parenchymatous cells, one of which will form the primary cambium, separate the primary phloem from the diarch xylem. The primary phloem is composed of sieve tubes, companion cells, and phloem parenchyma.

The pericycle is composed of one layer of rather irregularly shaped parenchymatous cells. It surrounds the primary phloem in a continuous sheath and abuts the protoxylem points. The cells are longer vertically than in their radial diameter. Lateral roots originate from the pericycle opposite the protoxylem points in all cases noted. The Casparian thickenings of the endodermis are fairly conspicuous, making this tissue readily recognizable. The cortex is composed of parenchymatous cells 10 to 12 layers in thickness. They are loosely arranged and irregular in shape. Intercellular

spaces are comparatively large in this tissue. The epidermis is one layer thick in the young root. The cells are very much greater in their vertical than in their radial dimension. The epidermis is the first tissue to differentiate out of the promeristem; followed closely by the cortex. No fibers are associated with the root or the hypocotyledonary bundles in the primary body.

THE TRANSITION REGION

The word "hypocotyl" as used in this paper refers to that morphological part of the plant between the cotyledonary node and the root. The hypocotyl may be easily located in many seedlings. Its

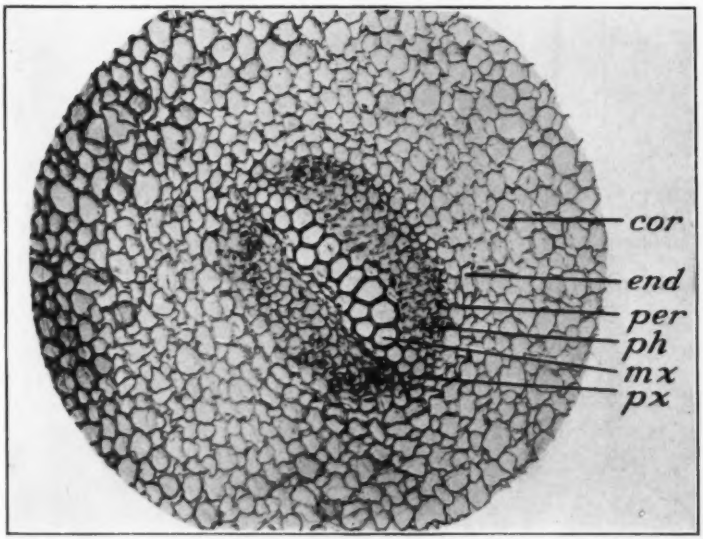

FIGURE 1.—Transverse section of young primary root; *px*, protoxylem; *mx*, metaxylem; *ph*, phloem, *per*, pericycle; *end*, endodermis; *cor*, cortex.

upper extremity is characterized by the divergence of the cotyledons. Its lower approximate terminus may usually be distinguished by the definite constriction which characterizes the beginning of the root structure. This is true in the case of the plant here described. The hypocotyl is not necessarily accompanied by anatomical changes throughout its entire length.

The term "transition region", as here used, refers only to that portion of the plant in which definite anatomical and histological changes take place in the reorientation of structures between root and stem. In many plants which possess a fleshly hypocotyl the transition region is located only in the upper portion of the hypocotyl. In the salsify the transition region is located entirely in the hypocotyl, although it includes only approximately the upper half of it, the lower portion being rootlike in anatomical structure.

The various changes occurring in the transition from root to stem structure of the primary body are given below, beginning with the

root and continuing through the hypocotyl to the base of the cotyledons where the transition is complete. The lower portion of the hypocotyl is diarch and rootlike in structure. Four primary xylem groups are present in the upper portion of the hypocotyl. The two primary xylem groups found in the root are continuous through

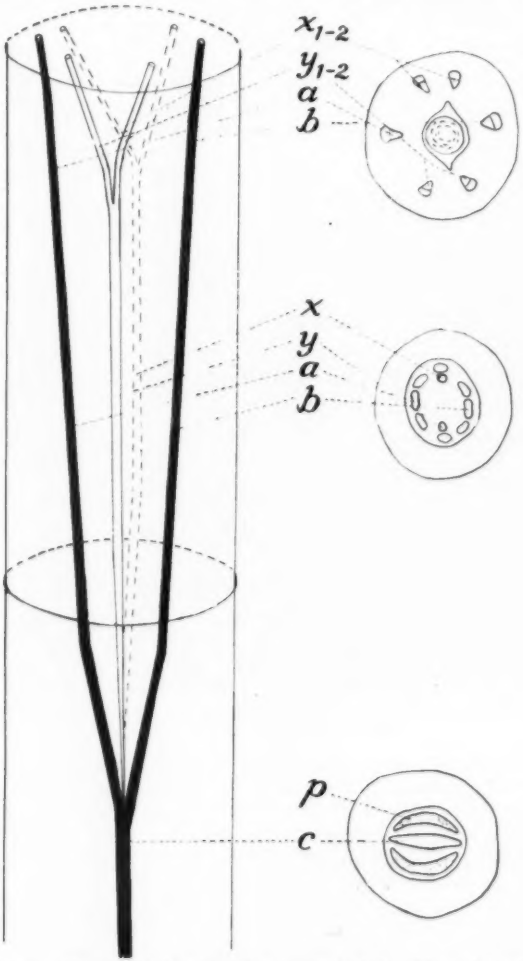

FIGURE 2.—Diagrammatic representation of vascular arrangement in transition region: *c*, Diarch xylem of lower region; *p*, primary phloem; *a*, *b*, cotyledonary bundles; *x*, *y*, lateral bundles; x_{1-2}, y_{1-2}, divergences of *x* and *y* respectively.

the whole length of the hypocotyl and diverge in their entirety into the cotyledons, making up, respectively, the midribs of each of the two sessile cotyledons. They are always on the same side as the cotyledons into which they diverge (fig. 2, *a* and *b*) and will hereafter be referred to as cotyledonary bundles.

Two bundles, in addition to the above-mentioned ones, arise in the hypocotyl. They are differentiated slightly later than the cotyledonary ones. In the upper portion of the hypocotyl these bundles are located laterally and at right angles with respect to the cotyledonary ones; hence they will hereafter be referred to as lateral bundles (fig. 2, *x* and *y*).

Some variation occurs in the origin of the lower portion of the lateral bundles in different individuals. In most instances the bundles are formed, in the lower transition region, from metaxylem which is continuous with that of the primary root (fig. 3). In some plants, however, the xylem is not continuous with the root, but originates at a higher level. In such cases pith cells separate the lower ends of the tracheae from those of the cotyledonary bundles and the lateral

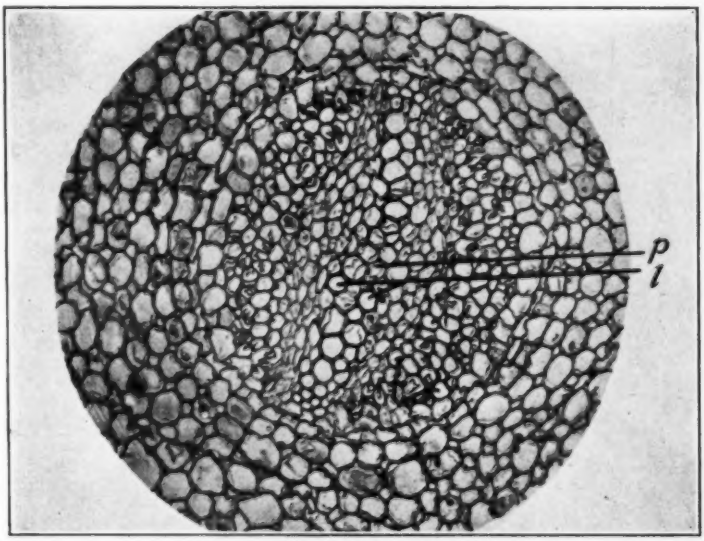

FIGURE 3.—Section through lower transition region showing origin of lateral bundles from metaxylem: *l*, Xylem of lateral bundles; *p*, pith.

bundles are differentiated directly out of the pith tissue which separates the two cotyledonary bundles. When this occurs, the primary xylem of the lateral bundles has no direct vascular connection with that of the diarch root. The primary phloem of all the bundles is continuous, however. There are also intermediate stages between these two situations. The metaxylem of the lower hypocotyl is sometimes continuous with that of the lateral bundles in a few strands only, with many pith cells interspersed among them. In other instances there are only a few pith cells, in which case several vessels of the lateral bundles are continuous with those of the lower hypocotyl and root.

At a slightly higher level the primary xylem of each of the cotyledonary bundles is farther apart, and pith is laid down between these two groups and thus also between xylem of the lateral bundles differentiating between them (fig. 4). More metaxylem is then differen-

tiated in this central mass, and finally two distinct groups appear. This xylem, at the level here described, is neither exarch nor endarch but forms the primary xylem of two transition bundles (fig. 5). In fact, these lateral bundles are never exarch or radial during their ontogeny. They are in a transition stage at the distal region from the cotyledonary node, and endarch collateral bundles in the upper portion of the hypocotyl. At first considerable parenchyma occurs between the xylem and phloem, but the xylem is laid down nearer and nearer to the phloem, until definite collateral bundles are organized. At this level there is no change in the exarch arrangement of the xylem in the cotyledonary bundles and they are still radial with

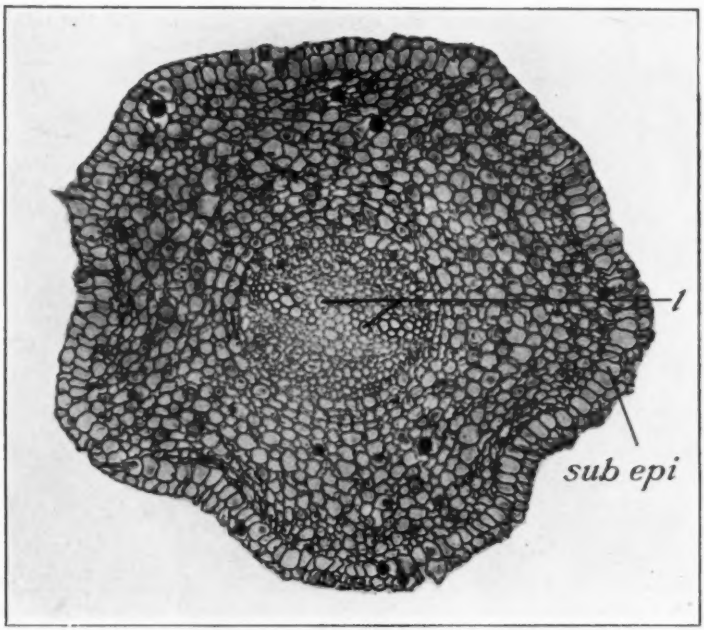

FIGURE 4.—Transverse section through lower transition zone. Note origin of lateral bundles (*l*) from metaxylem between cotyledonary bundles and also subepidermis (*sub epi*).

respect to the phloem. The pericycle is sometimes composed of two layers of cells opposite the protoxylem points at this position. Lateral roots originate from the pericycle opposite these points. The endodermis is structurally the same here as in the root. There are a few more layers of cortical cells, however. Two layers of epidermal cells begin to appear. Both layers are composed of large cells (fig. 5). The outer one, at its lower extremity, gives rise to numerous root hairs.

Farther up the hypocotyl the four bundles become more radially symmetrical, with a pith composed of loosely arranged parenchymatous cells. Within about 1 mm from the basal origin of the lateral bundles, all four of them are equally distributed at 90-degree intervals

from each other as one views them in transverse section. The lateral xylem groups are here collaterally arranged with respect to the two phloem groups. The phloem just outside of them is gradually reorientated until, at higher levels, it makes up the primary phloem of each of the lateral bundles. Here is found the first indication of transition of the primary cotyledonary bundles from exarch to endarch arrangement (fig. 7). The metaxylem is differentiated abaxially and on either side of the protoxylem. The protoxylem is then adaxially differentiated and thus through the center of the group of metaxylem cells. The lateral bundles are, at this level, still transition bundles. No appreciable change is evident in the position of the primary phloem.

At only a slightly higher level, and throughout most of the remaining upper hypocotyledonary region, the cotyledonary bundles are

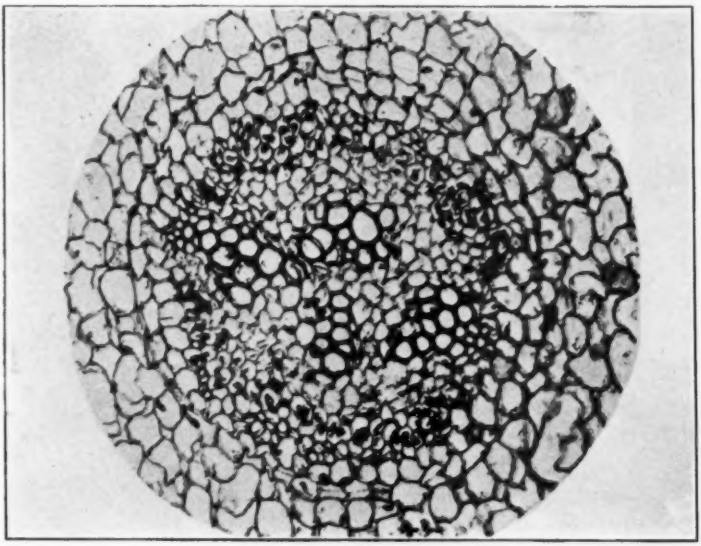

FIGURE 5.—Section through transition zone showing differentiation of two xylem groups between the cotyledonary bundles.

transition ones but are radial with respect to their primary xylem and phloem. At this level the xylem of the lateral bundles is more endarch than transitional and the bundles are nearly collateral. At a slightly higher level the cotyledonary bundles are transition radial ones and the two lateral bundles are perfectly endarch and collateral (figs. 6 and 7). Adaxial differentiation of the protoxylem has resulted in this endarch condition of the lateral bundles. In the cotyledonary bundles the abaxial differentiation of the metaxylem takes place laterally on both sides of the protoxylem. The pericycle at this level is two layers in thickness and the endodermis is still present but is irregular in shape.

At a position about 1 mm below the cotyledonary plate each phloem group associated radially with the cotyledonary bundles bifurcates,

forming two irregular groups. The two groups on either side and adjacent to the xylem of the cotyledonary bundles anastomose immediately outside of this xylem, and thus collateral bundles are formed. However, the xylem of these bundles is still in a transition stage. Small strands of the remaining groups of phloem, located between each cotyledonary and each lateral bundle, diverge into the cortex (fig. 8). A part of this phloem is continuous to a higher level, where it forms a ring of cells inside of the diverging primary vascular bundles. These phloem cells gradually disappear at higher levels

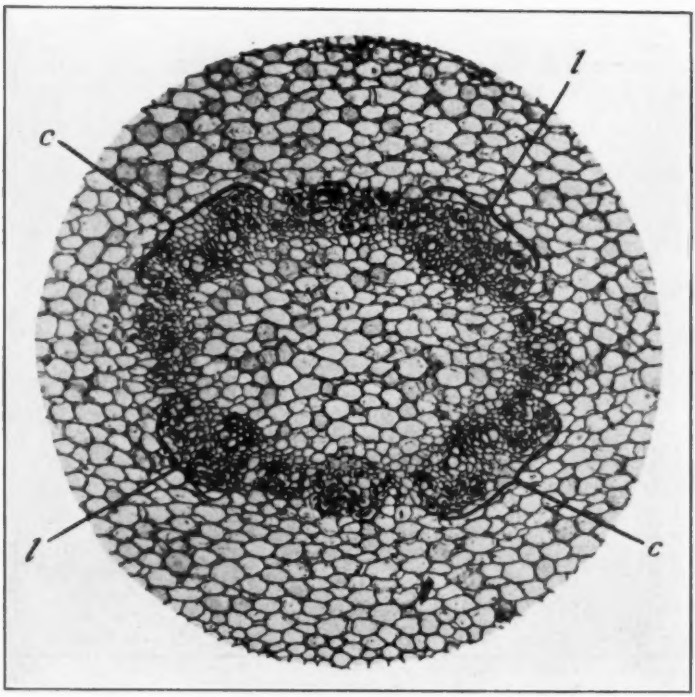

FIGURE 6.—Transverse section through upper hypocotyl. Note transition and radial arrangement of cotyledonary bundles (c) and endarch collateral position of lateral zones (*l*). × 100.

until the base of the epicotyl is reached, which is composed of a mass of undifferentiated meristemetic cells. The primary phloem of the root thus in part seems continuous with that of the stem. The primary xylem, however, is not continuous with that of the stem but diverges entirely into the cotyledons. Only the secondary xylem of the root is continuous with the xylem of the stem.

The xylem of the cotyledonary bundles is not endarch at the same level that the bundles are collateral but becomes so at a slightly higher level, and is entirely so at the position of the bundle divergence toward the cotyledons. At this same level each of the lateral bundles bifurcates, forming two closely associated bundles (fig. 9). These gradually are laid down farther apart until they finally form a lateral trace in

each of the two cotyledons (fig. 10). They always diverge and form the cotyledonary traces on the same side of the plant as that on which they arose and extend throughout their length in the hypocotyl. The cotyledonary bundles diverge at the same level and make up the midribs of the cotyledons.

Since salsify is definitely syncotyledonous, there is no sharp divergence of the cotyledonary traces, but they extend for some distance through the cotyledons in an almost vertical position.

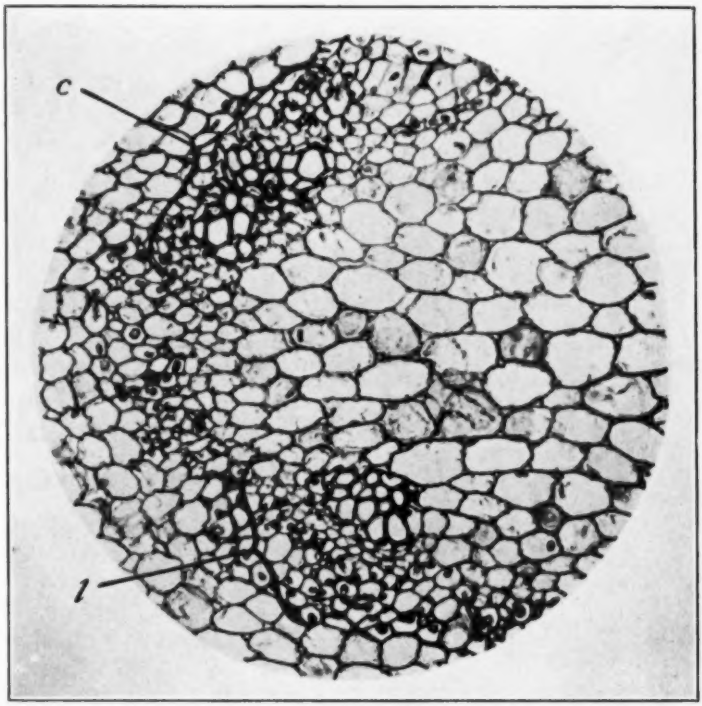

FIGURE 7.—Higher magnification of section shown in figure 6; note same points there suggested. × 250

ADDITIONAL OBSERVATIONS

In connection with the foregoing studies, it was noted that no oil ducts are present in either the young primary or the mature salsify. The substance commonly spoken of as "milky" is found mainly in the phloem.

The edible portion of the mature salsify is composed largely of xylem. Most of this is xylem parenchyma. The phloem, pericycle, endodermis, and cortex are also present in the mature structure. In striking contrast to the situation in such plants as the radish, carrot, and beet, in which the cork cambium develops from the pericycle, in the salsify it develops from cortical tissue. The endodermis is readily distinguished in the mature structure by the prominent Casparian strips.

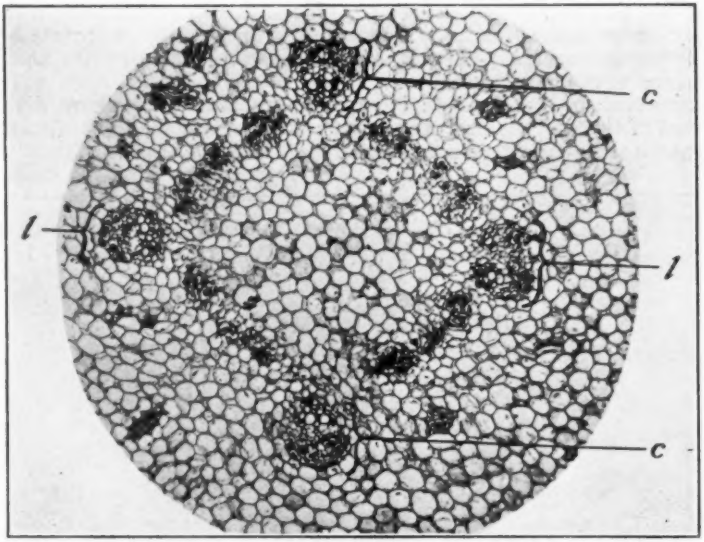

FIGURE 8.—Transverse section through upper portion of hypocotyl. Note primary phloem between the bundles and strands of it through the cortex: *l*, Lateral bundles; *c*, cotyledonary bundles.

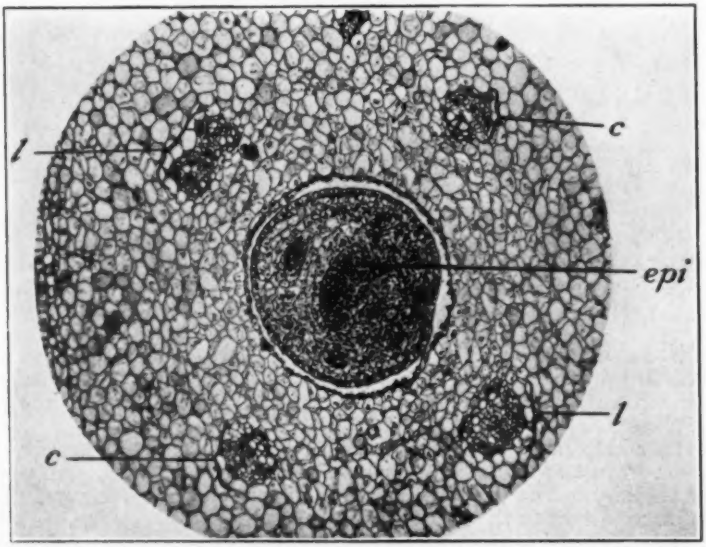

FIGURE 9.—Transverse section at base of epicotyl; lateral bundles slightly bifurcated and all bundles diverging into cotyledons: *l*, Lateral bundles; *c*, cotyledonary bundles; *epi*, base of epicotyl.

SUMMARY

The salsify seedling has linear, lanceolate, sessile cotyledons which are photosynthetic. The slowly developing epicotyl is enclosed in a cotyledonary tube to about 1 cm above the cotyledonary node.

The transition phenomena were studied from the root through the hypocotyl to the bases of the cotyledons where the transition is complete.

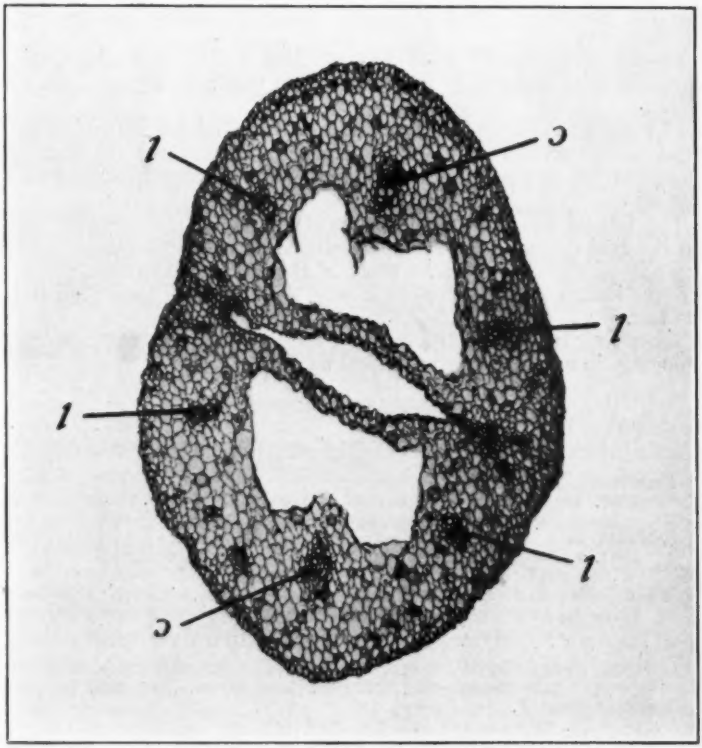

FIGURE 10.—Transverse section through cotyledons. Note lateral bundles become lateral traces of the cotyledons, while cotyledonary bundles become midribs of the cotyledons: *l*, Lateral bundles; *c*, cotyledonary bundles. The central cavity represents the cotyledonary tube; the larger cavities show the breaking down of the central parenchyma of the cotyledons.

The transition from root to stem structure occurs entirely in the hypocotyl.

The root is diarch and the primary xylem and phloem are radially arranged as is typical in the primary structure of roots.

The transition region is largely tetrarch; two primary bundles, in addition to the two continuous with those of the root, being differentiated in the lower portion of the transition zone from metaxylem.

Both the phloem and the xylem in the two primary bundles of the root are continuous through the hypocotyl and into the midribs of the cotyledons.

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The two additional bundles arising in the basal portion of the transition region are located laterally and at right angles with respect to the cotyledonary bundles.

Each lateral bundle separates near the cotyledonary node and the two strands thus formed make up a lateral trace in each of the two cotyledons.

The transition from the exarch radial arrangement of the primary cotyledonary bundles to the endarch collateral arrangement extends through the upper one-half of the hypocotyl, hence almost to the cotyledonary node.

During this transition the metaxylem vessels are differentiated abaxially with respect to the protoxylem and laterally on either side of it.

The primary cotyledonary bundles are radially arranged with respect to the xylem and phloem throughout most of the transition zone, but are typical collateral bundles where they diverge into the cotyledons.

The lateral bundles in the hypocotyl are never exarch or radial, but are laid down as transition bundles in the lower transition region and extend thus throughout most of this zone. However, there are endarch collateral ones at a lower level than those of the cotyledonary bundles.

These four bundles of the primary body extend through most of the hypocotyl in a radially symmetrical pattern.

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EFFECT OF INTERIOR TEMPERATURES OF BEEF MUSCLE UPON THE PRESS FLUID AND COOKING LOSSES[1]

By ALICE M. CHILD, *associate professor of home economics*, and J. AGNES FOGARTY, *Minnesota Agricultural Experiment Station*

INTRODUCTION

Quality and palatability of lean tissue of meat are largely dependent upon their physicochemical relationships. In examining the factors contributing to the edible qualities of meat, investigators have emphasized the importance of tenderness, juiciness, flavor, and aroma. Research dealing with these qualities has been effective in advancing scientific knowledge in meat cookery. Physicochemical investigation is necessary, however, to bring about a clear understanding of the changes that take place when meat is heated.

A method for studying the quality and quantity of press fluid or juiciness in different meat samples has been developed through the use of the pressometer (*4*).[2] The term "press fluid" is used to designate the fluid, consisting of moisture plus the soluble material plus the colloidal fraction, expressed from muscle by the use of the pressometer. This paper deals with a study of the quantity and composition of press fluid from beef muscle heated to different temperatures to determine their effect on the palatability of meat with respect to juiciness, a very important factor in meat quality.

REVIEW OF LITERATURE

The factors that affect the cooking losses of meat when roasted have been the subject of many investigations. Early work by Grindley and Mojonnier (*9*) dealt with the losses of meat cooked in water and by dry heat. Their results show that dry heat caused losses of from 0.25 to 4.55 percent of the nitrogenous matter and 2.47 to 27.18 percent of the fat. Cline and Godfrey (*5*) concluded that loss in weight varied directly with increase in temperature. Grindley and Emmett (*8*) studied the juice and fiber of meat cooked in different ways. The juice was removed from raw ground meat by pressure, the yield being approximately 30 cc per 100 g. On heating, the red juice changed to brown at 52° C. These investigators found that the juice had the distinctive flavor of meat, while the fibers had little or no flavor though prepared in different ways. The flavor of the juice was more pronounced in the liquid portion than in the coagulated precipitate. Cline, Trowbridge, Foster, and Fry (*6*) found, from judges' grading, that increased shrinkage was accompanied by a decrease in tenderness, juiciness, and flavor of lean meat and that the loss of flavor might be attributed to loss of juices.

[1] Received for publication Apr. 8, 1935; issued December, 1935. Scientific Journal Series, Paper No. 1343, Minnesota Agricultural Experiment Station.

[2] Reference is made by number (italic) to Literature Cited, p. 661.

Early investigation of pure muscle fluid—fluid removed from muscle not in contact with either water or salt—was carried on by Botazzi (*3*) in 1912. He obtained fluid from raw ox muscle that had been stored on ice and then chopped and ground with diatom powder. By means of a hydraulic press, using pressures varying from 50 to 350 atmospheres, he expressed the fluid in quantities sufficient for the determination of its physical and chemical properties. Because of the general inaccessibility of Botazzi's work, certain of his data are shown in table 1.

TABLE 1.—*Yield of press fluid, at 350 atmospheres pressure, from raw ox muscle, and dry residue, protein, and ash content of the fluid* [1]

Muscle	Weight of muscle	Weight of fluid	Yield	Dry residue	Total protein	Ash	Residue protein and ash
	Kilograms	*Kilograms*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Striated	0.938	0.529	63	8.914	91.086	1.739	2.654
Smooth	.829	.339	40	7.43	92.57	.85	2.93

[1] Botazzi (*3*).

Other analyses of fluid from ox muscle are given by Wiley (*14*), Leach (*11*), states that meat juice is the fluid portion of the muscle fibers. The standard for true meat juice adopted by the Association of Official Agricultural Chemists (*1*) is:

> The solids contain not more than 15 percent of ash, not more than 2.5 percent NaCl, not more than 4 nor less than 2 percent phosphoric acid, and not less than 12 percent of nitrogen. The nitrogenous bodies contain not less than 35 percent of coagulated proteins and not more than 40 percent of the meat basis.

Halliday, Noble, and Klaas (*10*), using a pressure of 3,800 pounds per square inch for expressing juice, found that rib roasts heated to 61° C. yielded a larger quantity of juice than rib roasts heated to 75°. The quantities of juice ranged about 15 cc per 100g. The juice from roasts heated to 61° was richer in solid and total nitrogen than that from roasts heated to 75°.

Bigelow and Cook (*2*) showed that a larger yield of juice could be obtained from meat heated to 60° C. than from raw muscle.

The methods and technique for press-fluid analyses, have been described by Child and Baldelli (*4*), who used 250 pounds pressure on the pressometer for 10 minutes. A study of sampling by Zohner [3] proved that duplicate samples from the same slice could be used for analysis, since the difference between them was not significant.

MATERIAL AND METHODS

The semitendinosus or eye muscle from the round of beef contains all of the structural components of striated muscle in a convenient compact unit. The entire raw cut weighs from 1 to 1.5 kg and measures from 25 to 30 cm in length and from 6 to 8 cm in diameter, being approximately cylindrical in shape and tapering abruptly at either end. The end pieces were not used for cooking. The muscle was removed from the round by cutting along the seam between the eye

[3] ZOHNER, E. T. A STUDY IN SAMPLING FOR PRESS FLUID INVESTIGATION. 1934. (Unpublished material, Minnesota Agricultural Experiment Station.)

and the top round and separating the muscle from the bottom round. The beef obtained from a large packing house was of choice quality and was ripened 14 days. An electric oven with an automatic control was used for cooking, and a pressometer was used for removing the fluid from the muscle.

PREPARING AND ROASTING THE MEAT FOR SAMPLING

Two roasts from the same muscle were prepared according to the method given in quality tests by the cooking committee of the cooperative meat investigation committee.[4] All exterior fat was carefully removed, leaving a continuous covering of connective tissue surrounding the muscle. Preliminary work showed that the meat was more uniformly cooked when prepared in this manner. The anterior portion was used for the tests at 58° C., and the posterior section for those at 75°. The length of the roast was determined by its diameter to insure even heat penetration to the center from all sides. The meat was cooked until the thermometer registered 58° for the rare and 75° for the well-done roasts. The former temperature was chosen because it is used for rare roasts by the cooking committee of the cooperative meat investigation committee.[4] The higher temperature was decided upon after a series of preliminary roasting experiments had been performed to determine the temperature at which the standard of the center slice of the roast would conform to that for well-done beef given by Sprague and Grindley (*13*), who describe the interior of meat cooked to 75° to be brownish gray in color, with a scant amount of colorless or slightly yellow juice.

Total losses, the sum of the evaporation and dripping losses, were obtained.

SAMPLING FOR PRESS-FLUID DETERMINATION

After cooking, the roasts were cooled to 40° C. They were then halved, and a slice 1.25 cm in thickness was removed from the center. On either side of the thermometer duplicate samples were taken by means of a round cylindrical borer 1.25 cm in diameter. The weighed sample was wrapped in an unsized, shrunk, weighed filter cloth and put in the tray of the pressometer under a pressure of 250 pounds for 10 minutes and weighed again. The weight of the press fluid was found by subtracting the weight of the pressed sample from the weight of the unpressed sample.

To obtain the quantity of press fluid per gram of dry matter, the expressed sample after preliminary evaporation in a drying oven was brought to constant weight at a temperature of 80° C. ± 2 under high vacuum. The ratio of press fluid to dry matter was calculated by dividing the weight of the press fluid by the weight of the dried sample.

OBTAINING PRESS FLUID FOR CHEMICAL ANALYSIS

For chemical analysis of the press fluid, the slice which had been sampled for press-fluid determination was used, the outer layer 1.25 cm in thickness being removed and the remainder cut into 1.25-cm cubes.

[4] ALEXANDER, L. M., CLARK, N. G., and HOWE, P. E. METHODS OF COOKING AND TESTING MEAT FOR PALATABILITY. Supplement to National Project Cooperative Meat Investigations. U. S. Dept. Agr., Bur. Home Econ. and Bur. Anim. Indus. 36 pp., illus. Revised, February 1933. [Mimeographed.]

The sample was tightly wrapped in a 5-cm square of filter cloth, placed on a perforated aluminum shelf, 1 cm high, in the pressometer tray, and kept under a pressure of 250 pounds for 5 minutes. Previous work had shown that during this period most of the nitrogen is removed (*4*). It was necessary to strain the press fluid when using raw meat in order to remove particles of fibers which were forced through the cloth by pressure. A saturated filter cloth square was used for this purpose.

Ten cubic centimeters of press fluid were collected for the determination of moisture, total nitrogen, and noncoagulable nitrogen in the press fluid. The fluid was kept at a temperature of 4° C. until analyzed. The percentage of moisture and of total and noncoagulable nitrogen in press fluid was determined by the method of the Association of Official Agricultural Chemists (*1*). The percentage of coagulable nitrogen was obtained by the difference between the means of duplicate analyses of total and noncoagulable nitrogen.

EXPERIMENTAL DATA

THE HOMOGENEITY OF SEMITENDINOSUS BEEF MUSCLE FOR PRESS-FLUID DETERMINATION

In a series of preliminary experiments portions of the semitendinosus beef muscle 5 cm apart were tested for uniformity of quantity and composition of press fluid, five muscles being used.

Three thermometers, 5 cm apart and approximately 5 cm from either end, were inserted in each muscle, which was cooked until the center thermometer registered 58° C., the end thermometers ranging between 59° and 60°. After the muscle was removed from the oven the rise in temperature varied from 1° to 3°. Press-fluid determinations were made on duplicate samples of slices taken from the anterior, center, and posterior parts of the muscle. Cooking losses were obtained from the roasts.

The results of this study showed that the differences in percentage of press fluid in each portion were so slight as to be negligible. The percentage of cooking losses likewise showed a very small deviation among the roasts.

A chemical analysis to determine the moisture, total nitrogen, and ether-extract content of the press fluid from the different portions was made. The composition of the press fluid in the muscle was found to be within the limits of experimental error. The ether extract was so small a percentage that this analysis was omitted in later work.

According to these analyses the semitendinosus beef muscle was uniform in chemical composition as well as in press-fluid yield; therefore, two comparable roasts were used from the same muscle.

PRESS FLUID IN SEMITENDINOSUS BEEF MUSCLE HEATED TO 58° AND 75° C.

Duplicate samples averaging 1.5 g were taken for the determination of press fluid from roasts from the same muscle. The anterior section was heated to 58° C. and the posterior to 75°.[5] The press fluid in muscles heated to 58° averaged 54.175 percent, with a standard deviation of 2.14; and that of muscles heated to 75° averaged 43.094 percent, with a standard deviation of 3.25.

[5] The temperatures 58° and 75° C. were reached in the oven. After removal, the rise in temperature of the 58° roast varied from 1° to 3°, but the temperature of the 75° roast remained constant.

The grams of press fluid per gram of dry matter were obtained by bringing the pressed sample to constant weight. The data obtained showed that the press fluid per gram of dry matter averaged 2.3186, with a standard deviation of 0.201 for the 58° muscles; and 1.4605, with a standard deviation of 0.248, for the 75° muscles.

Applying Fisher's (7) analysis of variance to the data on the percentages of press fluid and the grams of fluid per gram of dry matter, it was found that variation due to temperature was much larger than that due to error.

Data on both percentages of press fluid and grams of fluid per gram of dry matter show that there is a larger quantity of press fluid in muscle heated to 58° C. than in that heated to 75°.

When Fisher's (7) analysis of variance was applied to the data, a tendency was noted for the raw beef muscle to differ from the muscle heated to 58° and 75° C. in moisture, total nitrogen, and noncoagulable nitrogen (table 2).

TABLE 2.—*Means in percentage of moisture, total nitrogen, noncoagulable nitrogen and coagulable nitrogen in press fluid from raw beef muscle and the same heated to 58° and 75° C.*

Sample no.	Moisture			Total nitrogen			Noncoagulable nitrogen			Coagulable nitrogen		
	Raw	58°	75°	Raw	58°	75°	Raw	58°	75°	Raw	58°	75°
1	87.85	91.04	92.83	1.68	1.02	0.76	0.55	0.70	0.67	1.14	0.32	0.09
2	87.71	91.94	93.55	1.82	1.04	.74	.42	.55	.58	1.41	.48	.16
3	90.10	91.19	95.25	1.32	1.11	.54	.46	.70	.49	.86	.42	.04
4	88.61	91.51	93.57	1.57	1.05	.70	.50	.59	.66	1.07	.46	.01
5	88.25	90.63	94.05	1.63	1.08	.57	.47	.66	.51	1.16	.43	.06
6	89.98	90.14	93.65	1.36	1.18	.66	.48	.76	.62	.87	.42	.04
7	88.80	92.69	93.46	1.54	.89	.64	.43	.59	.57	1.11	.31	.08
8	87.58	90.45	93.68	1.25	1.05	.67	.48	.58	.63	.77	.47	.04
9	90.56	91.73	93.79	1.21	1.01	.59	.46	.63	.54	.75	.37	.05
10	87.43	91.16	92.16	1.61	1.06	.66	.49	.56	.61	1.12	.49	.05
11	87.45	91.83	93.66	1.58	1.03	.59	.48	.53	.54	1.10	.49	.05
12	87.38	92.23	94.26	1.68	.92	.60	.47	.63	.56	1.23	.30	.03
13	89.65	91.40	93.73	1.48	1.03	.68	.47	.66	.63	1.03	.49	.05
14	88.52	91.09	93.67	1.26	.89	.55	.39	.64	.50	.85	.24	.05
15	86.94	91.02	92.53	1.67	.86	.65	.41	.66	.65	1.24	.20	.01
16	90.16	91.80	93.33	1.26	.93	.64	.40	.61	.60	.87	.31	.05
Mean	88.56	91.37	93.57	1.49	1.01	.64	.46	.63	.58	1.04	.39	.05

The means of the different analyses show three distinct trends in the composition of the press fluid at each temperature. The moisture content of the press fluid varies directly with the interior temperature, being 93.57 percent in muscle heated to 75° C., and 91.37 percent in muscle heated to 58°. Raw muscle has the lowest percentage, 88.56. As the muscle proteins coagulate with increased temperature, the coagulable nitrogen fraction is removed, thus yielding a less concentrated press fluid.

The percentage of total nitrogen shows an inverse relationship with the temperature, 0.64 percent in the 75° muscle, and 1.01 percent in the 58° muscle. The raw muscle has the highest percentage, 1.49. The removal of the heat-coagulable nitrogen with increased temperature brings about this relationship.

The averages for the percentage of coagulable nitrogen in the press fluid follow the same order as for the total nitrogen, 1.04 percent for the raw, 0.39 percent for the 58°, and 0.05 percent for the 75°.

Coagulable nitrogen varies consistently with increase in temperature, little being present at 75°. The small quantity of coagulable nitrogen at 75° indicates that coagulation is practically complete at that temperature. The noncoagulable nitrogen content averages 0.46 percent for the press fluid from the raw muscle, 0.63 for the 58°, and 0.58 percent for the 75°.

These values indicate that the noncoagulable nitrogen from the 58° and that from the 75° muscle are only slightly different. Enzymes may be the influencing factor. The increased enzyme activity at 58° C. releases additional amino nitrogen which analyzes as non coagulable nitrogen. The higher temperature of 75° inhibits enzyme activity, thus decreasing the yield of noncoagulable nitrogen.

PRESS FLUID AND COOKING LOSSES

Press fluid and total cooking losses were obtained for roasts cooked to 58° and 75° C. interior temperatures. These data are given in table 3 (32 pairs of observations).

TABLE 3.—*Press fluid and total cooking losses in semitendinosus beef muscle heated to 58° and to 75° C.*

58° C.			75° C.		
Roast no.	Press fluid	Cooking losses	Roast no.	Press fluid	Cooking losses
	Percent	*Percent*		*Percent*	*Percent*
1	58.346	9.759	2	47.872	25.719
3	56.416	10.836	4	44.315	24.752
5	53.347	19.230	6	41.950	30.688
7	52.693	9.136	8	42.675	24.483
9	54.203	15.000	10	51.218	32.626
11	54.946	13.810	12	41.830	32.409
13	56.282	12.376	14	41.088	34.708
15	52.960	11.261	16	44.415	31.463
17	53.274	11.807	18	41.968	30.857
19	55.059	12.619	20	46.214	30.081
21	54.910	10.976	22	40.612	30.417
23	53.875	10.304	24	41.858	35.345
25	55.534	11.491	26	42.262	31.330
27	55.363	13.400	28	39.726	32.200
29	50.814	14.050	30	38.950	37.846
31	54.284	11.258	32	45.356	30.045
33	53.185	19.001	34	39.184	36.657
35	55.814	14.167	36	37.890	34.342
37	55.308	12.224	38	48.843	29.768
39	51.284	13.907	40	42.382	29.791
41	54.060	13.062	42	45.410	28.831
43	53.380	11.811	44	39.066	37.021
45	55.176	15.385	46	43.843	32.321
47	55.806	10.938	48	44.404	32.588
49	51.410	11.111	50	45.345	26.038
51	55.416	14.471	52	46.116	34.156
53	49.222	13.937	54	42.768	29.392
55	53.074	9.388	56	45.703	22.303
57	56.65	11.957	58	45.602	31.070
59	54.094	14.566	60	46.692	27.588
61	54.887	10.358	62	47.006	25.086
63	52.782	11.849	64	41.786	33.108

The value for the correlation coefficient r is -0.5331 when correlating percentage of press fluid and total cooking losses from the 75° C. roasts. This value is highly significant, since the least highly significant value ($P=0.01$) for r is 0.4487.

The value for r in the correlation between percentage cooking losses and press fluid in the roasts cooked to 58° C. is -0.1823, and

the least significant value ($P=0.05$) for r is 0.3494. No significant relationship is indicated between total cooking losses and press fluid in roasts cooked to 58°. According to McCance and Shipp (*12*), muscle tissue shortens without a change in volume or loss of weight when heated to 40°. However, at temperatures above 60° there is a loss of weight caused by increased shrinkage of the meat proteins, causing expression of the meat juices. This may explain the inverse relationship observed when muscle is heated to 75° and the fact that there is no definite relationship at 58°.

SUMMARY

From this study the following observations on the effect of interior temperature upon the semitendinosus muscle of beef can be made on the basis of statistical analysis.

The ratio of press fluid to dry matter is greater in muscle heated to 58° C. than in that heated to 75°, the value for grams of press fluid per gram of dry matter being 2.319 for the 58° muscle and 1.46 for the 75°.

Approximately 11 percent more press fluid is found in the muscle heated to 58° than in that heated to 75°.

Chemical analysis of the press fluid showed:

The moisture content of press fluid varies directly with the interior temperature, the raw having less moisture than the heated muscle.

An inverse relationship exists between the total nitrogen content of press fluid and the interior temperature, the raw having more total nitrogen.

There is comparatively little difference in the noncoagulable nitrogen in press fluid from muscles heated to 58° and 75° C., the raw having less than the heated muscle.

The coagulable nitrogen fraction in press fluid varies inversely with the interior temperature.

An inverse relationship exists between the percentage of press fluid and the total cooking losses in muscle heated to 75° C. No relationship is indicated between the percentage of press fluid and the total cooking losses in muscle heated to 58°.

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THE DIFFUSIBLE CALCIUM IN THE SERUM OF LAYING AND NONLAYING HENS [1]

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INTRODUCTION

The calcium metabolism of fowls is of considerable theoretical interest because of their ability to absorb and to deposit in the eggshell a large amount of calcium. It is, also, of interest that the hen is able to tolerate readily, as compared with mammals, an extremely high level of calcium in the blood. In an effort to obtain facts which might serve to explain this high tolerance for calcium, determinations were made of the diffusible and nondiffusible calcium in the blood serum of laying and of nonlaying hens. It seemed particularly desirable in these studies to ascertain whether there were any changes in the relative amounts of these two calcium fractions when the hen passed from a nonlaying into a laying status and vice versa. Therefore, the determinations were made on serum from a small group of hens before they began to lay and after they began to lay.

REVIEW OF LITERATURE

Since the completion of this work, reports on the same subject have appeared from other laboratories. Correll and Hughes (*3*),[2] comparing cocks, laying hens, and nonlaying hens, found the ultrafiltrable calcium of the serum to be fairly constant at a value of 6.4 mg, although the total calcium varied from 11.7 to 25.1 mg per 100 cc. Their analyses were carried out according to the method of Nicholas (*7*), with cellophane as the semipermeable membrane. Laskowski (*5*), using collodion membranes, found the ultrafiltrable calcium to be 8.0 mg per 100 cc in the case of both laying and nonlaying hens. However, this value was obtained on plasma rather than on serum. Benjamin and Hess (*1*), using a collodion membrane and relatively low pressure differences, obtained a value of 6.8 mg per 100 cc of serum for laying hens. It is unfortunate, for the purposes of comparison, that the methods used in these investigations and in ultrafiltration and diffusion studies in general have varied considerably. However, the results obtained by different investigators on avian serum are in essential agreement.

EXPERIMENTAL METHOD

The methods available for this type of study may be classified in three groups: (1) Ultrafiltration, in which the serum is forced through a semipermeable membrane by high or low pressure; (2) pressure

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[2] Reference is made by number (italic) to Literature Cited, p. 667.

dialysis, in which the sample is separated from pure water or a salt solution by a semipermeable membrane, a difference in pressure being maintained between the two sides of the membrane; and (3) ordinary dialysis. The second method was chosen for these studies since it appeared to lend itself readily to the handling of small samples.

The apparatus used for the pressure dialysis of the serum was essentially that described by Moritz (*6*). The negative pressure was obtained by means of a suction pump and was regulated at 150 mm±10 mm of mercury by means of a mercury escape valve. It was found necessary to include a large reservoir in the circuit to avoid wide fluctuations in pressure.

Collodion sacs were prepared according to a method based on the work of Farmer (*4*) and Brown (*2*). A solution of 10-percent Parlodion in equal parts of absolute ethyl alcohol and diethyl ether was used. A test tube of appropriate size was filled with collodion, emptied, and drained for exactly 1 minute, as described by Farmer (*4*). Then, with the open end of the test tube up, the film was dried for exactly 1 minute by lowering a capillary tube, to which suction was applied, nearly to the bottom of the test tube. The test tube was then immersed in 95-percent ethyl alcohol and allowed to remain for at least 5 minutes. The alcohol was applied to harden the membrane and to give high permeability. Several investigators have used alcohol for this purpose, particularly in the preparation of membranes of graded permeability; the higher the concentration of alcohol used for hardening, the greater the permeability. The sacs were then removed from the tubes, washed with distilled water, and preserved for future use in normal saline solution. Chloroform was added as a preservative. It is important that the sacs should not be allowed to dry since drying greatly reduces the permeability.

In carrying out a determination, the following procedure was adopted: Three cubic centimeters of distilled water was measured into a 15-cc centrifuge tube and the tube and contents weighed. A sac from the saline solution was washed with distilled water and dried inside and out with filter paper, and 2 cc of serum was then measured into it. In practice it was found easiest to slip the sac onto its supporting stopper before adding the serum, and then to add the serum by inserting the tip of the pipette through the hole of the stopper. The sac was then secured to the stopper by means of an encircling thread and the whole pushed onto the glass tube which opened to the atmosphere. These operations had to be carried out quickly to prevent any lessening of the permeability of the sac by drying. After the complete apparatus had been assembled and when suction was first applied, the sac was held above the level of the water in order to observe possible leaks. It was then lowered so that the level inside the sac was just below that on the outside. The dialysis was carried on for 4 hours, the sac being raised occasionally to maintain the relative levels of the liquids.

At the end of the dialysis the sac was again raised above the level of the outside liquid and the suction slowly released. The apparatus was separated, care being taken to transfer as much as possible of the liquid adhering to the outside of the sac to the centrifuge tube. The latter was then weighed, the gain in weight being the measure of the water which had left the sac. No correction was found to be necessary for the amount of water adhering to the sac since several trials showed

it to be negligible. The outside of the sac was then washed into the tube containing the dialysate, and the solution within the sac was washed out into a second centrifuge tube. Calcium was determined in both of these by the method of Kramer and Tisdall as modified by Tisdall (*8*). From these results the diffusible calcium was calculated in the way described by Updegraff, Greenberg, and Clark (*9*). The total calcium concentration was obtained by adding the amount of calcium in the dialysate to that which remained in the sac. The accuracy of this calculation was found to be within the limits of error of the method for calcium determination.

Six nonlaying hens which later began to lay were employed in this experiment. Blood samples of about 30 cc were obtained from the heart by means of a hypodermic needle and syringe at approximately monthly intervals. The blood serum was separated by centrifuging.

RESULTS AND DISCUSSION

Since trouble was experienced in preparing highly permeable membranes, a number of determinations were carried out on a solution of $Ca(H_2PO_4)_2.H_2O$ containing 18 mg of calcium per 100 cc. Under these conditions, 90.9 ± 1.4 percent of the calcium was diffusible through the various membranes. While theoretically 100 percent of the calcium in an entirely inorganic solution should be diffusible, it is doubtful whether this would be the case under the conditions of this experiment. Thus, it is probable that a greater degree of diffusibility would have been found had the dialysis period been longer. Furthermore, duplicate tests on serum showed close agreement even though the permeability of the particular membranes used in the tests varied widely when used with the inorganic calcium solution.

The results obtained on the dialysis of serum are summarized in table 1. From these figures it may be seen that although the total calcium level of the serum varied from 10.6 to 39.0 mg per 100 cc, the diffusible calcium was maintained within a much narrower range, all the values except two falling between 4.2 and 6.7, with an average for all determinations of 5.3 ± 0.17 mg. This value is lower than the 6.4 reported by Correll and Hughes (*3*) or the 6.8 reported by Benjamin and Hess (*1*). The differences may be due to the fact that the aforementioned investigators used ultrafiltration methods, while the method employed in these experiments was pressure dialysis. The significantly higher value, 8.0 mg, which Laskowski (*5*) obtained on plasma is not strictly comparable with the results obtained on serum. It may be concluded, therefore, that for any given method of analysis the so-called "ultrafiltrable" or diffusible calcium of fowl serum shows a high degree of constancy which is quite independent of the total calcium concentration of that serum. Benjamin and Hess (*1*) and others have shown that this is not true in cases of other species in which the level of calcium in the blood has been elevated artificially or pathologically. It is apparent, also, that slight changes in the procedure, at least in this particular type of analysis, may give constant differences in results.

TABLE 1.—*Total and diffusible calcium of hen's serum*

Bird no.	Month and day of analysis	Condition of bird	Determinations	Total calcium [1]		Diffusible calcium [1]	
				Range	Mean	Range	Mean
			Number	*Mg*	*Mg*	*Mg*	*Mg*
123	Jan. 12	Nonlaying	4	9.9–11.3	10.6	6.3–6.6	6.5
	Jan. 26	Laying	5	20.2–21.7	21.3	6.1–6.5	6.3
	Feb. 16	do	2	25.5–25.5	25.5	5.0–5.4	5.2
	May 3	do	3	17.3–20.0	18.9	3.9–4.8	4.2
274	Jan. 12	Nonlaying	5	11.3–11.9	11.6	5.8–6.5	6.2
	Jan. 27	Laying	5	23.5–24.7	23.8	5.5–6.6	6.0
	Feb. 16	Nonlaying	4	11.8–12.1	11.9	3.9–4.9	4.5
177	Jan. 15	do	4	11.9–12.3	12.1	6.5–6.8	6.7
	Jan. 29	Laying	4	38.2–39.9	39.0	5.1–6.2	5.6
	Feb. 26	do	5	16.5–20.4	18.8	3.1–4.6	3.7
	Jan. 19	do	5	24.8–26.5	25.9	5.7–7.4	6.5
372	Feb. 9	do	4	18.5–19.3	18.9	4.3–5.1	4.6
	Apr. 19	do	4	21.8–22.1	21.9	4.2–4.4	4.3
	Jan. 19	Nonlaying	4	12.5–13.4	12.7	5.6–6.8	6.1
923	Feb. 9	Laying	4	17.2–18.9	17.9	6.1–6.7	6.4
	Apr. 19	do	3	16.5–16.5	16.5	4.9–5.2	5.0
	Jan. 21	do	5	15.3–16.1	15.6	5.1–6.1	5.7
117	Feb. 11	Nonlaying	2	11.7–12.7	12.2	2.5–2.9	2.7
	May 3	Laying	3	13.4–14.5	14.1	4.3–5.5	5.1
10	Jan. 5	Nonlaying; on rachitogenic diet	6	12.6–13.3	12.9	7.9–8.5	8.2

[1] Per 100 cc of serum.

As indicated earlier in this paper, the birds were in a nonlaying condition at the beginning of the experiment, but each one began to lay before or soon after the first set of analyses had been made. In some instances there occurred a further change back to the nonlaying condition prior to the last analysis. Although in each case the total calcium increased markedly with the advent of the laying period, in no case was there a significant change in the value of the diffusible calcium. Furthermore, the average value for all analyses made while the birds were not laying, 5.4 ± 0.42 mg per 100 cc is not significantly different from the value 5.3 ± 0.17 mg per 100 cc obtained when they were laying. The identity of these values seems particularly significant because all the values were determined on serum from the same group of birds. It is generally agreed that the nondiffusible calcium of serum exists in some kind of combination with the protein. Benjamin and Hess (*1*) have further divided the diffusible and nondiffusible fractions according to whether or not they are absorbed by dry $BaSO_4$. They find, as do others, that a rise in the total calcium of the blood is generally accompanied by a rise in serum protein. The possibility that the nondiffusible calcium exists as a calcium-protein complex may explain the tolerance of laying hens for an extremely high level of blood calcium. It is not known whether the calcium in this complex is available for the formation of eggshell, but apparently it has little toxic activity.

Bird no. 10 had been on a rachitic diet for 9 months previous to the assay. It was killed by decapitation and the blood sample collected during the subsequent bleeding. The value 8.2 mg per 100 cc of serum for the diffusible calcium, while it may be due to the method of bleeding, is believed to be indicative of the disturbed calcium metabolism caused by the rachitic diet.

SUMMARY

By the use of collodion membranes, the diffusible calcium of the serum of nonlaying hens was found to be 5.4 ± 0.42 and that of the same hens in the laying condition 5.3 ± 0.17 mg per 100 cc of serum. No difference was observed in the level of diffusible calcium when the birds changed from a nonlaying to a laying condition or vice versa. The nondiffusible calcium in the serum rose from a value of 6.4 ± 0.53 mg per 100 cc for nonlaying birds to a value of 16.1 ± 1.17 mg per 100 cc for the same birds in the laying condition.

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